University of Sussex

# Effects of Virulence and Fraction 1 antigens from *Yersinia pestis* on the human innate immune system

A Thesis By

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# ***ABSTRACT***

***Yersinia pestis*, the aetiological agent of plague, is responsible for a disease that has killed over 200 million people throughout history and generated three pandemics. This bacterium's terrible success in causing disease is owed greatly to the virulence factors it expresses. Two of these factors are V antigen (LcrV) and F1 antigen (Caf1), both of which are two major antigens which the immune system produces antibodies against. V antigen is already known to have vital roles in *Y. pestis* gene expression and translocating other virulence factors into the host cells as well as having some immunosuppressive effects while F1 antigen is better known for possessing an antiphagocytic effect. The effects that these two antigens have in modulating the innate immune system of Mono Mac 6 cells were studied, such as modulation of expression of pattern recognition receptors (PRRs), in particular Toll-like receptors (TLRs), activation of NF-κB and secretion of cytokines, particularly those involved in inflammatory responses, as well as localising where in the cell these antigens target to. It was demonstrated that both V and F1 antigens possess immunosuppressive abilities, such as downregulation of TLRs as well as inhibitition of NF-κB activation and suppression of secretion of the cytokines TNF-α, IL-6 and IL-10. Furthermore, stimulation with only either V or F1 antigens can upregulate expression of the scavenger receptor CD36 and are capable of inducing secretion of the anti-inflammatory cytokine IL-10. V and F1 antigens were found to localise in the Golgi apparatus 30 minutes after stimulation and it was also determined that these antigens interfere with the signalling molecule MyD88.**

# *Chapter 1 - Introduction*

## 1.1. The Immune System

The human immune system can be broadly classified into the adaptive and the innate immune systems (Janeway, 2001). Adaptive immunity mediates a delayed, specific response to foreign antigen while innate immunity is not antigen specific and develops immediately following exposure to immune stimuli (Janeway & Medzhitov, 2002). Adaptive immune responses are essential for control of pathogens that escape elimination by the innate immune response and are important in development of immunological memory. Because of its role in immune memory, the adaptive immune systems' contributions to pathogen elimination and vaccine development have been widely studied. The innate immune system was long thought to be a non-specific inflammatory response generated during exposure to foreign antigen. However, studies conducted in recent years indicate the innate immune response is able to discriminate between pathogen classes and direct innate and adaptive immune responses toward elimination of the invading pathogen. The discovery of pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) and the role they play in elimination of pathogen and activity as adjuvant has renewed interest in the importance of the previously neglected innate immune system (Janeway, 1989; Medzhitov, et al., 1997).

### 1.1.1. Innate Immunity

The Immunologists' dirty little secret:

*"..In order to obtain readily detectable responses to (protein) antigens, they must be incorporated into a remarkable mixture termed complete Freund's adjuvant. The most likely possibility (to explain this) is that primitive effector cells bear receptors that allow recognition of certain* ***pathogen-associated molecular patterns*** *that are not found in the host. I term these receptors* **pattern recognition receptors**. *I propose that these pattern recognition systems activated effector functions of primitive immune systems before the development of rearranging gene families and continue to play a role in host defense today…"*

Charles Janeway, 1989
"Approaching the Asymptote? Evolution and Revolution in Immunology
Cold Spring Harbor Symposia on Quantitative Biology, 1989, 54 Pt 1: 1-13

Almost twenty years ago, Charles Janeway changed our view of the innate immune system, by publishing "Approaching the Asymptote?" as part of the Cold Spring Harbor Symposium on immune recognition.

In this publication he predicted that there would be molecules that were encoded in the *germ line* which would recognize the presence of molecules produced by broad classes of pathogens. He called these molecules pattern recognition receptors (PRRs) and the ligands that they recognise, pathogen-associated molecular pattern (PAMPs) (Janeway, 1989).

Janeway's prediction that the immune system is dependent upon evolutionarily conserved pathogen pattern recognition receptors was dramatically realized almost 10 years later, when in 1996, Jules Hoffman and colleagues published the paper for which Hoffman received the Nobel (Nobelprize.org, 2011), showing that a receptor called "Toll" was essential for fruit flies to mount an immune response against fungi (Lemaitre, et al., 1996).

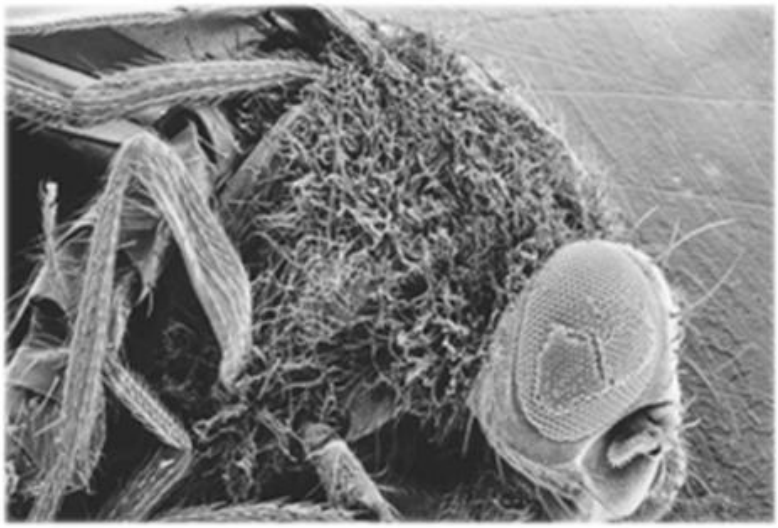

**Figure 1.1 –** Scanning electron micrograph of germinating hyphae of *Aspergillus fumigates* on a dead Drosophila which has succumbed to the infection (200x magnification) **(Lemaitre, et al., 1996)**.

In 1997, Janeway's lab cloned and characterized the first of these receptors, human Toll, Toll-like receptor 4 (TLR4) (Medzhitov, et al., 1997). In 1998, Bruce Beutler's group made the seminal discovery that TLR4 is the receptor for bacterial lipopolysaccharide (LPS) (Poltorak, et al., 1998), which finally demonstrated that TLRs are able to sense microbial structures. Beutler received the Nobel Prize for this discovery (Nobelprize.org, 2011) and sparked an onslaught of research in the field of innate immunity.

Known PRR families include: the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), the RIG-like receptors (RLRs) and the C-type lectin receptors (CLRs) (Kawai & Akira, 2009; Mogensen, 2009). Other PRRs include the protein kinase RNA-activated (PKR) and the intracellular DNA sensors absent in melanoma 2 (AIM2) and DNA-dependent activation of IFN-regulatory factors (DAI) (Mogensen, 2009). Each of these receptors is responsible for sensing microbial signatures and triggering the innate immune response (Kawai & Akira, 2009; Mogensen, 2009).

#### 1.1.1.1. Toll-like Receptors (TLRs)

During the 1980s, researchers in Germany found that developing flies (*Drosophila melanogaster*) could not establish a proper dorsal-ventral axis without a transmembrane signal receptor protein dubbed Toll (meaning 'weird' in German slang) (Kindt, et al., 2007). In 1996, Jules Hoffman and Bruno Lemaitre found that mutated Toll caused flies to be highly susceptible to lethal infection with a fungus to which wild-type flies were immune (Lemaitre, et al., 1996) and, in 1997, Ruslan Medzhitov and Charles Janeway discovered a protein in humans, which shared homology between its cytoplasmic domain and that of Toll, that activated the expression of immune response genes, the first evidence that an immune response pathway was conserved between fruit flies and humans (Medzhitov, et al., 1997). This protein later became known as Toll-like receptor (TLR) 4. In 1998, studies with mutant mice in the laboratory of Bruce Beutler proved that TLRs are part of the normal immune physiology of mammals and that TLR4 is indispensable for the recognition of lipopolysaccharide (LPS) (Poltorak, et al., 1998). Since then, the work of many investigators has determined that there are many TLRs (Kindt, et al., 2007).

##### 1.1.1.1.1. Structure of TLRs

Toll-like receptors (TLRs) are membrane-spanning glycoproteins which possess three domains: extracellular or endosomal domain, transmembrane domain and intracellular or cytoplasmic domain. They share a common structural motif in their extracellular or endosomal region, which is predicted to look like a horseshoe-like structure. These motifs are called leucine-rich repeats (LRRs). All TLRs contain several LRRs and a subset of the LRRs make up the extracellular ligand-binding region of the TLR. The TLR intracellular domain is called the Toll/IL-1 receptor (TIR) domain, named thus due to the similarity between the cytoplasmic domains of TLRs and an IL-1 receptor. These TIR domains possess three regions, highly conserved among all members of the TIR family, that serve as binding sites for the intracellular proteins that participate in the signalling pathways of TLRs (Mogensen, 2009; Kawai & Akira, 2009). To date, 10 functional TLRs have been identified in humans (TLR1-10) and 12

functional TLRs in mice (TLR1-9 and TLR11-13). TLR10 is not functional in mice due to a retrovirus insertion, which substitutes the C-terminal half of the receptor for an unrelated and non-productive sequence in the *Tlr10* gene, and TLR11-13 are lost in the human genome, with the *Tlr11* gene having a stop codon that results in a lack of production of the receptor (Takeda & Akira, 2005; Kawai & Akira, 2009; Mogensen, 2009).

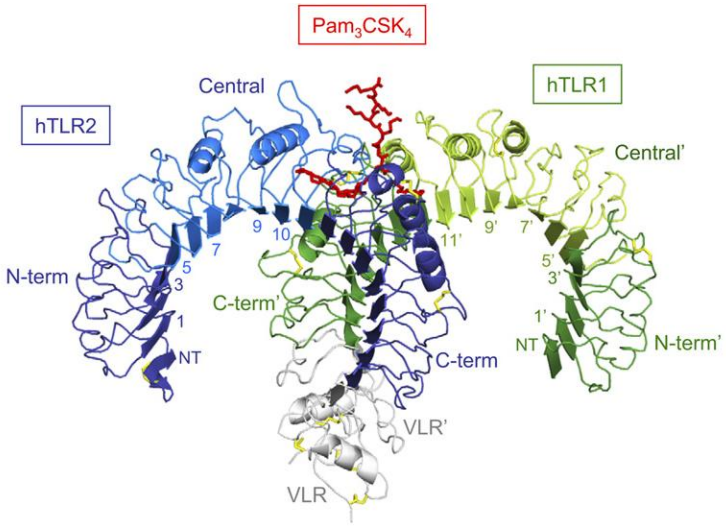


**Figure 1.2 –** The horseshoe-shaped crystal structure of the human TLR2 (blue)/TLR1 (green) heterodimer associated with the tri-acylated lipopeptide $Pam_3CSK_4$ (red). TLRs have amino-terminus (N-term) leucine rich repeats (LRR) for ligand binding and a carboxy-terminus (C-term) intracellular Toll/interleukin-1 receptor (TIR) homology domain for signal transduction **(Jin, et al., 2007)**.

1.1.1.1.2. TLR ligands

TLRs are considered the most important group of PRRs discovered to date. These receptors allow cells, particularly dendritic cells and macrophages, to detect a broad spectrum of viruses, bacteria, fungi and even some protozoa (Kindt, et al., 2007). TLRs may recognize their respective PAMP either directly (e.g. TLR3 binds directly to double-stranded RNA) or via an intermediary PAMP-binding molecule and/or another receptor (e.g. TLR4 recognizes ‘smooth’ LPS via the accessory molecule MD2, LPS-binding protein (LBP) and the GPI-linked protein CD14). The ability of different regions of the extracellular domain of TLRs to bind to their respective PAMPs or different PAMP-binding molecules may be what allows certain TLRs to recognize

structurally and biochemically unrelated ligands, such as TLR4's ability to recognize bacterial LPS and viral fusion protein from respiratory syncytial virus (RSV). TLR2's ability to form a heterodimer with TLR1, TLR6, CD14, CD36 or others allows for further distinction between different PAMPs. This broad recognition of PAMPs by all the TLRs (and even the other PRRs) means that no single class of pathogen is recognized by only one TLR (or PRR) but rather by various TLRs via various PAMPs in pathogens (Mogensen, 2009; Kawai & Akira, 2009).

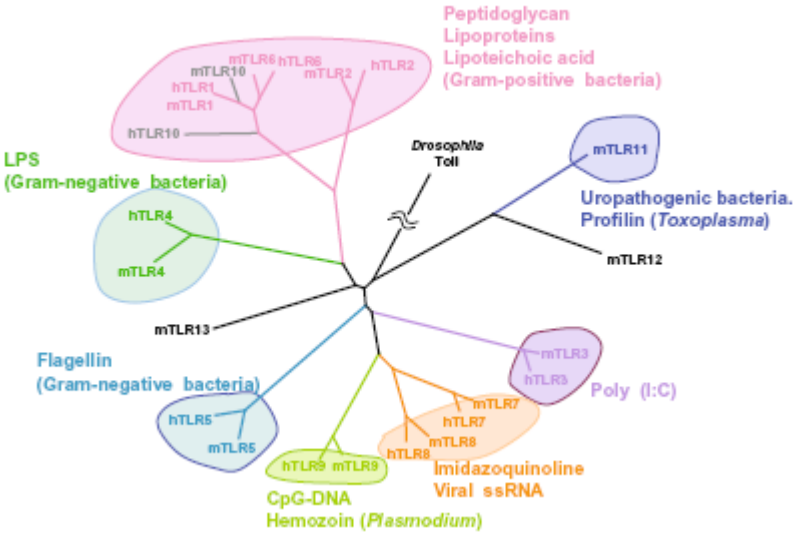


**Figure 1.3 –** Phylogenic classification of murine (m) and human (h) TLRs **(Kumagai, et al., 2008)**.

**Table 1.1 –** Toll-like receptors and the ligands they recognize **(Kindt, et al., 2007; Playfair & Bancroft, 2008; Kawai & Akira, 2009; Mogensen, 2009)**.

| **Receptor** | **Recognized ligands (source of ligand)** |
|---|---|
| TLR1/TLR2 | Triacyl lipopeptides (mycobacteria) |
| TLR2 | Peptidoglycans (Gram-positive and –negative bacteria) |
| | Lipoproteins (various pathogens, e.g. mycobacteria) |
| | GPI-mucin (Trypanosomes) |
| | Lipoarabinomannan (mycobacteria) |
| | PI dimmanoside (mycobacteria) |
| | Zymosan (fungi) |
| | Porins (*Neisseria*) |
| | Envelope glycoproteins (e.g. measles virus, HSV, cytomegalovirus) |
| | Phospholipomannan (*Candida*) |
| | β-Glycan (fungi) |
| TLR3 | Double-stranded RNA (dsRNA) (viruses) |
| TLR4 | Lipopolysaccharide (LPS) (Gram-negative bacteria) |
| | Envelope glycoproteins (viruses, e.g. RSV) |
| | Glycoinositolphospholipids (protozoa) |
| | Mannan (*Candida*) |
| | HSP70 (host) |
| TLR5 | Flagellin (flagellated bacteria) |
| TLR6/TLR2 | Diacyl lipopeptides (mycobacteria) |
| | Zymosan (fungi) |
| | Lipoteichoic acid (LTA) (Gram-positive bacteria) |

| **Receptor** | **Recognized ligands (source of ligand)** |
|---|---|
| TLR7 | Single-stranded RNA (ssRNA) (RNA viruses)<br>Imidazoquinolines (antiviral drugs) |
| TLR8 | Single-stranded RNA (ssRNA) (RNA viruses)<br>Imidazoquinolines (antiviral drugs) |
| TLR9 | Unmethylated CpG DNA (bacteria, DNA viruses, protozoa)<br>Haemozoin (*Plasmodium falciparum*)<br>Herpesvirus infection (some herpesviruses) |
| hTLR10 | ? |
| mTLR11 | Undetermined (uropathogenic bacteria)<br>Profilin-like molecule (*Toxoplasma gondii*) |
| mTLR12 | ? |
| mTLR13 | ? |

Typically, TLRs that recognize extracellular ligands, mostly related to bacteria, are present on the surface of cells (TLR1, TLR2, TLR4, TLR5, TLR6, human TLR10 and mouse TLR11) while TLRs that recognize intracellular ligands, mostly related to viruses, are found in intracellular compartments called endosomes (TLR3, TLR7, TLR8 and TLR9) (Mogensen, 2009; Kawai & Akira, 2009). The intracellular TLRs are normally expressed on the endoplasmic reticulum in resting cells but are trafficked to the endosomal compartment in response to PAMP-mediated stimulation. The localization of these intracellular TLRs in endosomes is important for the recognition of viral nucleic acids that are delivered to these vesicles as well as for discrimination of self from nonself nucleic acids, thus preventing responses caused by recognition to host nucleic acids (Kawai & Akira, 2009). In tissues, the most important cell types that express TLRs are antigen-presenting cells, which include macrophages, dendritic cells

and B-cells. However, TLRs have been found in most cell types, either expressed constitutively or expressed in an inducible manner during infection (Mogensen, 2009).

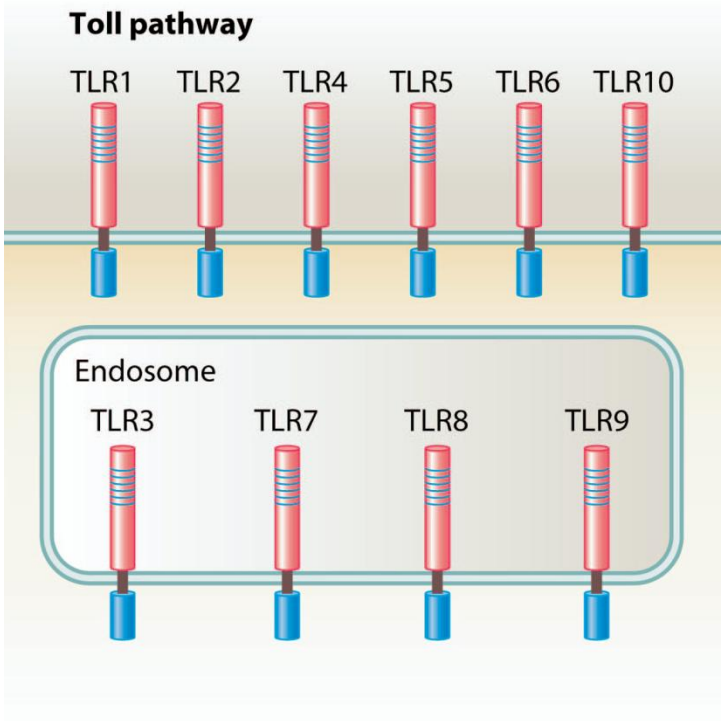


**Figure 1.4 – Location of TLRs in the cell.** TLRs located on the surface of the cell (TLR1, 2, 4, 5, 6 & 10) and within endosomes (TLR3, 7, 8 & 9). A TLR has an extracellular domain (red with blue lines), a transmembrane domain (brown) and a cytoplasmic domain (blue) **(Mogensen, 2009)**.

Activation of TLR signalling pathways has many effects. It promotes expression of genes that contribute to inflammation, induce changes in antigen-presenting cells that make them more efficient and cause the synthesis and secretion of intercellular signalling molecules that affect the behaviour of leukocytes. On macrophages, once these TLRs recognize their respective PAMP and the signalling pathways are activated, they stimulate phagocytosis as well as production of chemical agents, such as molecules toxic to phagocytosed microbes and cytokines (Kindt, et al., 2007).

1.1.1.1.3. TLR signalling

While TLRs and their roles in innate immunity are still considered to be recent discoveries, the signalling pathways used by TLRs have already been mapped. Once a

ligand binds to a TLR, it dimerizes, often forming homodimers although sometimes, as previously mentioned in the case of TLR2 with either TLR1 or TLR6, as heterodimers. The TLR dimer undergoes conformational changes that are necessary for the subsequent recruitment of cytosolic TIR domain-containing adaptor molecules. The TIR domain offers binding sites for proteins which also contain TIR domains, known as adaptor proteins (Kawai & Akira, 2009; Mogensen, 2009).

The adaptor used by most TLRs is myeloid differentiation primary-response protein 88 (MyD88) and the signalling pathway involving this adaptor is known as the MyD88-dependent pathway. However, TLR3 does not use this adaptor protein while TLR4, while it does use this pathway, also uses a second pathway, the same utilized by TLR3, called the MyD88-independent, TRIF-dependent pathway (Kindt, et al., 2007; Mogensen, 2009). Both TLR3 and TLR4 are bound to by an adaptor molecule known as TIR domain-containing adaptor inducing IFN-β (TRIF) (Mogensen, 2009).

Other adaptors involved with certain TLR signalling cascades include MyD88 adaptor-like protein (MAL) and TRIF-related adaptor molecule (TRAM). MAL appears to be necessary to act as an intermediary molecule between most TLRs and MyD88, with the exception of TLR5, -7, -8 and -9 in which MyD88 appears to bind directly to their TIR domain. TRAM acts as an intermediary molecule between TLR4 and TRIF but not TLR3 which appears to bind directly to TRIF. Recruitment of one or several adaptor proteins to an activated TLR is followed by activation of downstream signal transduction pathways via phosphorylation, ubiquitination or protein-protein interactions. A fifth adaptor protein has been identified for TLRs, sterile alpha- and armadillo motif-containing protein (SARM), which is unlike the other adaptors in that it does not conduct a signalling cascade but rather negatively regulates TRIF-dependent signalling in human cells. The existence of this family of adaptors and the selective use of individual adaptors or combinations of adaptors may be part of the answer on how different responses are induced by different TLRs (Mogensen, 2009).

TLR4, which uses both MyD88-dependent and MyD88-independent pathways, activates them sequentially. First, it starts MyD88-dependent signalling on the cell surface and afterwards, TLR4 is internalized and trafficked to the endosome where it activates its MyD88-independent pathway (Kawai & Akira, 2009).

1.1.1.1.3.1. MyD88-dependent signalling pathway

In MyD88-dependent pathways stimulated through TLR2, -4 and -5, MyD88 primarily drives the inflammatory gene expression, while in signalling triggered from TLR7, -8 and -9, MyD88-dependent pathways also stimulate type I IFN production (Mogensen, 2009).

In a typical MyD88-dependent pathway, once MyD88 binds to either the TIR domain of a TLR or to MAL, which is itself bound to the TIR domain of a TLR, it promotes the association of the protein kinases IL-1 receptor (IL-1R)-associated kinase 4 (IRAK4) and IRAK1/2. In the IRAK4:IRAK1/2 complex, IRAK4 phosphorylates IRAK1/2, creating a docking site on IRAK1/2 for TNF-receptor-associated factor 6 (TRAF6). TRAF6, which acts as a ubiquitin protein ligase (E3), binds and then dissociates together with IRAK1/2 from IRAK4. The IRAK1/2:TRAF6 complex, together with the E2 ubiquitination enzyme complex (Ubc13 and Uev1A), catalyzes the synthesis of polyubiquitin chains on TRAF6 itself and other substrates, including another protein kinase, transforming growth factor (TGF)-β-activated kinase 1 (TAK1), and the IκB kinase (IKK) subunit NF-κB essential modifier (NEMO). The ubiquitinated TRAF6 then recruits TAK1-binding protein 2 (TAB2) and TAB3, which brings TAK1 into proximity of the signalling complex and activates TAK1 kinase activity. Activated TAK1, in complex with TAB1, TAB2 and TAB3, is the crossroads to two other pathways: mitogen-activated protein kinase (MAPK) pathway and NF-κB pathway (Kindt, et al., 2007; Mogensen, 2009; Kawai & Akira, 2009).

The activation of the MAPK pathway occurs when TAK1 phosphorylates and activates members of the MAPK kinase (MKK) family, including MKK3, -4, -6 and -7. MKK3/6 then phosphorylate and activate p38 while MKK4/7 phosphorylate and activate c-Jun N-terminal kinase (JNK). These signalling pathways ultimately lead to activation of the transcription factor activator protein 1 (AP-1). AP-1 enters the nucleus and promotes phosphorylation of one or more transcription factors and therefore activates transcription of MAPK-dependent genes, which then affect the cell cycle or cellular differentiation (Kindt, et al., 2007; Mogensen, 2009).

The NF-κB family consists of five members that can exist as dimers. The heterodimer composed of RelA and p50 is considered to be the most frequently activated transcription factor of this family in PRR signalling (Kawai & Akira, 2009). NF-κB is a powerful transcription factor that is inhibited by inhibitor of nuclear factor NF-κB (IκB) in its unphosphorylated form, which sequesters bound NF-κB in the cytoplasm. The NF-κB pathway is activated by TAK1 phosphorylating IKK, which is composed of the subunits IKKα, IKKβ and NEMO (also known as IKKγ). When TAK1 activates IKK, the latter kinase phosphorylates IκB. The phosphorylated IκB then undergoes proteosomal degradation, causing the release of NF-κB which can then translocate to the nucleus. In the nucleus, NF-κB binds to κB sites present in promoters and enhancers of certain genes, which initiates the transcription of many genes necessary for the effector functions of innate immunity (proinflammatory genes) (Kindt, et al., 2007; Mogensen, 2009). In vertebrates, this means production of cytokines, adhesion molecules and other effectors (Kindt, et al., 2007).

1.1.1.1.3.2. MyD88-independent signalling pathway

In the MyD88-independent or TRIF-dependent pathway, triggered from TLR3 or TLR4, TRIF, associated with TRAM in the case of TLR4, initiates this signalling pathway. Following this, it acts upon TRAF3 and TRAF family member-associated NF-κB activator (TANK) to activate the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε, which mediate direct phosphorylation of IFN regulatory factor 3 (IRF3) and IRF7. Due to this phosphorylation, IRF3 and IRF7 form hetero- or homodimers, translocate to the nucleus and, in association with transcriptional coactivators such as CBP and p300, initiate transcription of IFN and IFN-inducible genes by binding to target sequences in DNA, such as IFN-stimulated response elements, driving type I IFN expression (Mogensen, 2009).

TLR7 and -9 also trigger IFN production, primarily in plasmacytoid dendritic cells. This is done through a molecular network including IRAK1, IRAK4, TRAF6, TRAF3 and IKKα, which results in the IRAK1-mediated phosphorylation of IRF7 and production of type I IFN (Mogensen, 2009).

TRIF-dependent activation of NF-κB occurs when TRIF recruits TRAF6 as well as receptor-interacting protein 1 (RIP-1) and TNF receptor (TNFR)-associated death domain (TRADD), which form a complex together with TRAF6 after RIP-1 is polyubiquitinated. These two molecules work together to facilitate activation of TAK1 and, therefore, the NF-κB pathway as described previously (Mogensen, 2009; Kawai & Akira, 2009).

Furthermore, when IRFs, NF-κB and AP1 meet in the nucleus, they can form a multiprotein complex, known as the enhanceosome, which induces transcription of the IFN-β gene (Mogensen, 2009).

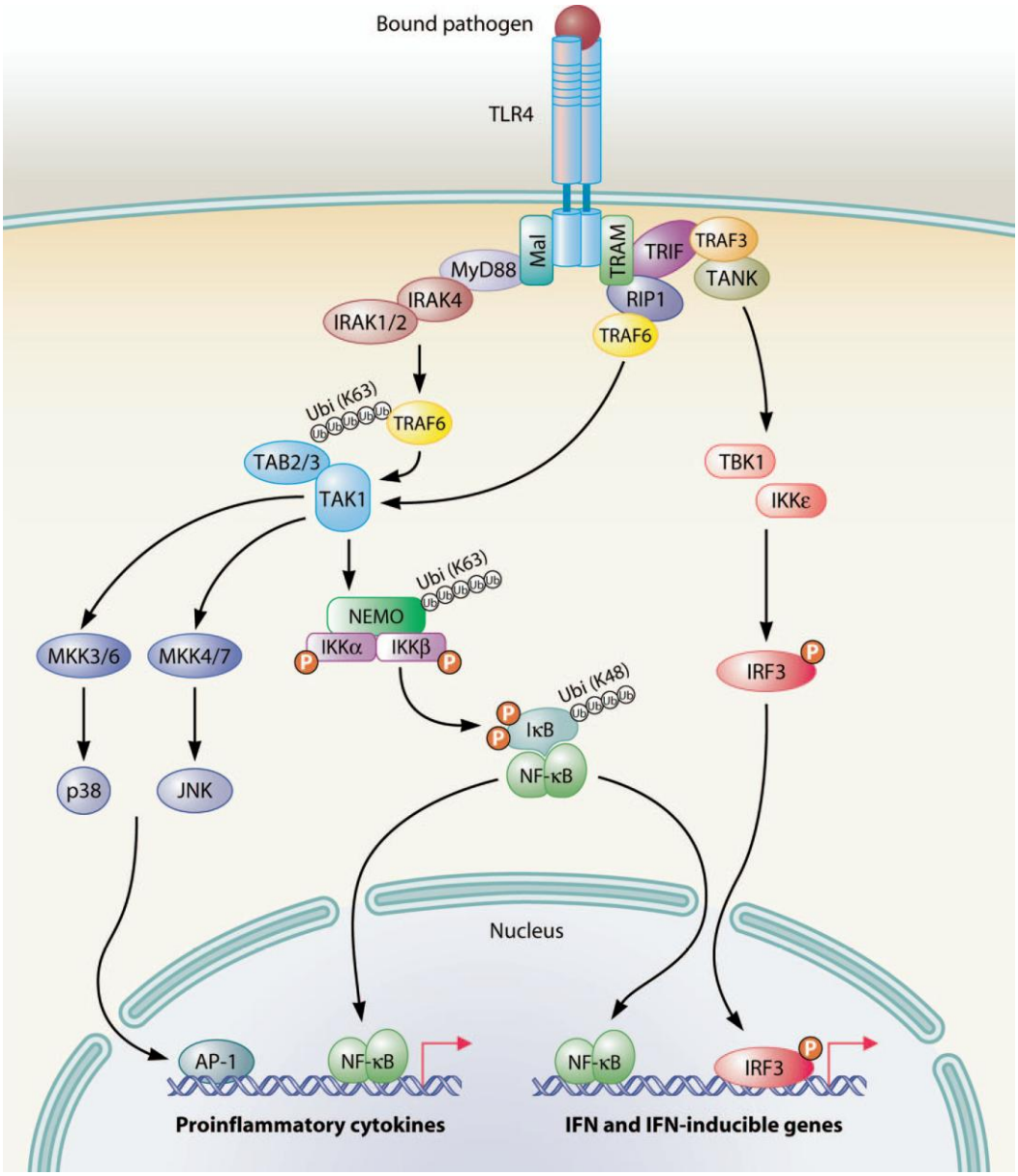


**Figure 1.5 – TLR signalling pathways.** MyD88-dependent pathway (seen on the left-hand side) and TRIF-dependent pathway (seen on the right-hand side) **(Mogensen, 2009)**.

1.1.1.1.3.3. Regulation of TLR signalling

Tight regulation of TLR activation and signalling as well as gene expression is essential to ensure that the strong nature of these responses is kept under control and transient. Excessive or inappropriate inflammation can be harmful to the host and result in immunopathology or autoimmunity. Therefore, at later stages after infection,

restriction and downregulation (e.g. expression of TLRs) needs to occur as needed within the cells. Some of the regulatory mechanisms are posttranscriptional regulatory mechanisms, particularly control of mRNA stability and degradation by the use of RNA-destabilizing proteins. Other mechanisms include the sequential induction of activating and inhibitory signals after activation of TLR signalling (Mogensen, 2009).

These inhibitory signals include several cellular molecules that negatively regulate TLR signalling. One of them, the fifth TLR adaptor molecule SARM, has been mentioned previously. Another molecule is MyD88s, an alternatively spliced variant of MyD88 which does not bind to IRAK4, thus does not promote IRAK1/2 phosphorylation and consequent NF-κB activation. Single immunoglobulin IL-1R-related molecule and ST2 regulate TLR signalling by sequestering MyD88 and Mal. IRAK-M is another inhibitory molecule, which is of the IRAK family but is deficient in kinase activity and therefore abrogates further downstream signalling. There is also the family of suppressor of cytokine signalling (SOCS) proteins, which interfere with cytokine-induced JAK-STAT signalling and are inducible by both cytokines and TLR ligands. The deubiquitinating enzymes A20 and CYLD have been attributed key roles in terminating TLR responses by a mechanism involving deubiquitination of TRAF6. The tripartite-motif protein family member TRIM30a induces degradation of TAB2/3 required for TAK1 activation and function, thereby preventing downstream signalling to NF-κB. Another regulatory mechanism is offered by the NF-κB-mediated induction of IκB gene expression, whose product, IκB, sequesters free activated NF-κB (Mogensen, 2009). Many more inhibitory molecules exist asides from these examples (Kawai & Akira, 2009). In short, there are a whole host of molecules involved in inhibiting TLR signalling, with the possibility of more which may yet be found than these mentioned.

Another prominent regulatory mechanism of immunity occurs when prior exposure of immunity cells induces a transient state of unresponsiveness towards a second stimulus. This state of unresponsiveness, characterized by the lack of an adequate proinflammatory cytokine response, is what is known as tolerance. When this involves TLRs, it is referred to as TLR tolerance (Reithmeier-Rost, et al., 2004). There are three types of TLR tolerance observed. When a first stimulus with a tolerizing PAMP occurs and then a second stimulus with a challenging PAMP that binds to a

different TLR than the first occurs and generates tolerance, this is called heterotolerance. When the tolerizing PAMP and the challenging PAMP, both different, utilize the the same TLR and generate tolerance, this is referred to as homotolerance and autotolerance is when both the tolerizing PAMP and the challenging PAMP are actually the same (Reithmeier-Rost, et al., 2004).

#### 1.1.1.2. Dysregulated Innate Immune Responses

Regulation of the innate immune system is important to keep the body alive. A loss of regulation or excessive stimulation of innate immunity can lead to dangerous and even fatal symptoms (Mogensen, 2009). When septicaemia occurs, that is, when bacteria infect the bloodstream, this can lead to an overreaction of the innate immune system and even into sepsis, the failure of one or more organs caused by this overreaction. Certain microbial molecules, such as LPS, can cause havoc in the immune system by overstimulating macrophages to release massive amounts of cytokines, such as TNF-α and IL-1. The complement system is also massively activated which, with the consequent inflammation, can damage small blood vessels and, together with the accumulation of PMNs, lead to fluid leaking into the alveoli of the lungs (adult respiratory distress syndrome, one of the most dreaded complications of severe injury and/or infection). The widespread blood clotting and overactivation of the fibrinolytic pathway, coupled with the resulting low blood pressure and decreased oxygen supply, lead to the vital organ failure and vascular collapse of septic shock (Cohen, 2002; Margetic, 2012). Other examples of diseases caused by loss of regulation include several inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Papakadis & Targan, 2000), rheumatoid arthritis due to excessive local cytokine secretion (Li, et al., 2009), and hereditary angioedema due to low levels of the complement protein C1 inhibitor (Playfair & Bancroft, 2008).

Thus it is of particular importance to identify molecules that are able to modulate innate immune responses. Certain virulence factors from bacteria have shown to be able to modulate innate immune responses and it is the focus of this thesis to characterise two such virulence proteins from *Yersinia pestis*.

## 1.2. *Yersinia* Species

*Yersinia* species are enterobacteriaceae, a heterogeneous group of Gram-negative aerobic bacilli. Enterobacteriaceae are also known as coliforms or enteric bacteria. *Yersinia* species exhibit bipolar staining and are short, pleomorphic rods and facultatively anaerobic. Most in this genus have animals as natural hosts but a few can produce serious disease in humans. The genus *Yersinia* consists of 11 species, of which 3 are human pathogens: *Yersinia pestis*, the aetiological agent for plague, *Y. pseudotuberculosis* and *Y. enterocolitica*, important causes of human diarrhoeal diseases (Perry & Fetherston, 1997; Brooks, et al., 2004; Elliot, et al., 2007).

*Y. pseudotuberculosis* is primarily an animal pathogen (domestic and farm animals and birds) although it can occasionally cause mesenteric adenitis in humans. More rarely it can cause septicaemia. It is likely transmitted to humans via food contaminated with infected animal faeces. *Y. enterocolitica* causes diarrhoeal disease, terminal ileitis and mesenteric adenitis. Infection may be complicated by septicaemia or reactive polyarthritis. Transmission may occur via faecal-oral route (e.g. contaminated food) from domestic (sheep, cattle, swine, dogs and cats) and wild animals (rodents) (Brooks, et al., 2004; Elliot, et al., 2007).

*Y. pestis* is a non-motile, Gram-negative, facultative intracellular coccobacillus that exhibits striking bipolar staining with specific stains (such as Wayson's stain or Wright-Giemsa stain). It is the cause of plague and is still endemic in some areas of the world, such as India, Southeast Asia (especially Vietnam), Africa and North and South America (including the western states of the USA and Mexico). It is primarily a pathogen found in rodents (rats, field mice, gerbils, moles, skunks and others) and is transmitted to humans via infected fleas, especially the rat flea (*Xenopsylla cheopis*). It grows at temperatures of 4 to ~40$^{o}$C and tolerates pH 5 to 9.6 but its optimal growth occurs at temperatures of 28 to 30$^{o}$C and pH 7.2 to 7.6 (Perry & Fetherston, 1997; Brooks, et al., 2004; Elliot, et al., 2007). Unless quickly treated, bubonic plague can have a mortality rate of 50% to 90% and septicaemic and pneumonic plague can have nearly 100% mortality (Salyers & Whitt, 2002; Rollins, et al., 2003; Brooks, et al., 2004; Prentice & Rahalison, 2007). Diagnosis and appropriate treatment reduces bubonic and septicaemic plague mortality to 5% to 15%. Although very rare, strains of

*Y. pestis* with antimicrobial resistance have been found, worryingly, in transferrable plasmids (Prentice & Rahalison, 2007).

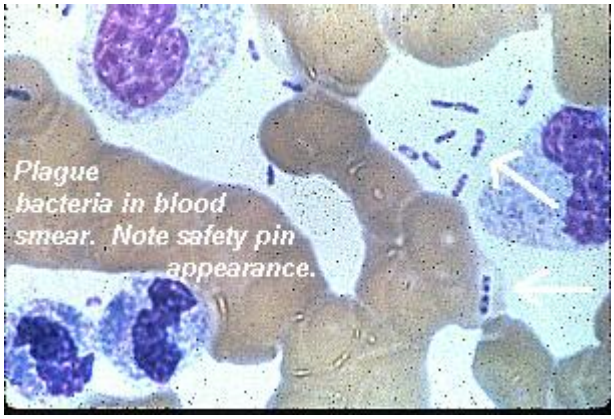


**Figure 1.6 –** *Yersinia pestis* seen in a blood smear (CDC, 2009).

1.2.1. Plague

The plague is a disease that has come to stand as a paradigm for infectious disease disasters. While this position is not entirely deserved, with other diseases having killed far more people and still being problematic diseases today, plague still has a special mystique (Salyers & Whitt, 2002). Interest in this disease was renewed after the events of the 11$^{th}$ of September of 2001, the subsequent distribution of *B. anthracis* spores through US mail and the news of two New Mexico residents visiting New York being diagnosed with bubonic plague in November of 2002, which sparked fears of a new bioterrorism attack (Rollins, et al., 2003).

The first recorded outbreak of plague was the Plague of the Philistines, in 1320 B.C. This plague is referenced in the Bible in I Samuel 5:6. Three known pandemics of plague have occurred throughout human history. The first recorded plague pandemic, known as the Justinian plague began with an epidemic in Pelusium, Egypt in 541 A.D., after arriving from Ethiopia, ending in 544 A.D. The epidemic quickly spread from there through the Middle East and the Mediterranean basin and, more limitedly, into Mediterranean Europe. Afterwards, the other epidemics occurred, from 558 to 767 A.D., and spread the pandemic throughout the then ‘known world’ – North Africa,

Europe and central and southern Asia (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Rollins, et al., 2003; Josko, 2004; Haensch, et al., 2010).

The second pandemic, famously known as the Black Death occurred in the 1300s and spread from Asia to Europe, where it raged for 100 years before subsiding. Just prior to the beginning of the second pandemic, plague probably spread from the steppes of central Asia and travelled westward along the trade routes from 1330 to 1346. In 1347, the appearance of plague in Sicily marked the start of the first epidemic of the second pandemic, which lasted until 1347. It spread throughout Europe, killing an estimated 17 million to 28 million Europeans (30 to 40% of European population at the time). Following this epidemic, other epidemics occurred throughout Europe and are responsible for the majority of the deaths due to plague of the entire pandemic. In England, plague epidemics occurred in cycles from 1361 to 1480. The second pandemic lasted in Europe until 1750 (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Rollins, et al., 2003; Josko, 2004; Haensch, et al., 2010).

The third pandemic likely began in Yunnan province, in China, in 1855, eventually spreading all over the world. Plague reached Hong Kong and Canton in 1894, Bombay in 1898, and was then disseminated by steamships from 1899 to 1900, reaching Africa, Australia, Europe, Hawaii, Japan, the Middle East, the Philippines, the United States (where it first appeared in San Francisco in March 1900) and South America. In India alone, 12.5 million people are thought to have died of plague between 1898 and 1918. This pandemic was finally considered over in 1950, likely due to effective public health measures as well as the emergence of antibiotics but not before establishing stable enzootic foci on every major continent except Australia (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Rollins, et al., 2003; Josko, 2004; Haensch, et al., 2010).


**Figure 1.7 –** Routes taken by the three recognised plague pandemics and the proposed origins of these pandemics (circled numbers). 1 – First pandemic (Justinian plague); 2 – Second pandemic (Black Death); 3 – Third pandemic **(Prentice & Rahalison, 2007)**.

More recently, two epidemics of plague have occurred in western India, from September to October 1994, proving that plague is not an eradicated disease but can still cause problems nowadays (Perry & Fetherston, 1997). At least 1000 to 3000 cases of plague worldwide are still reported annually to the World Health Organization (WHO) (Titball, et al., 2003; Josko, 2004). In 2000 and 2001, more than 95% of reported cases were from the African region, with 41% being from a well-known focus in Madagascar alone (Prentice & Rahalison, 2007). Plague is estimated to have killed 100 to 200 million people throughout history, making it one of the leading infectious disease killers of human beings (Rollins, et al., 2003).

It was during the third pandemic, during the Hong Kong epidemic in June 1894, that Alexandre Yersin announced the isolation of the plague bacterium. He also developed an antiserum against plague, using it to cure a plague patient in 1896, and made the connection between rats and plague (Perry & Fetherston, 1997; Rollins, et al., 2003). It was a French colonial Army physician in Indochina, Paul-Louis Simond, who identified the flea as the vector for the plague bacterium (Rollins, et al., 2003). *Yersinia pestis* has undergone many name changes. It was originally known as *Bacterium pestis* until 1900, then *Bacillus pestis* until 1923, *Pasteurella pestis* (after Yersin's mentor)

until 1970, when it was renamed a final time as *Yersinia pestis* (Perry & Fetherston, 1997).

### 1.2.2. *Y. pestis* Virulence Factors

*Y. pestis'* success in causing these pandemics lies in the fact that it possesses several plasmids that encode virulence factors including a 70 to 75-kbp plasmid (pCD1) that is essential for its virulence and common to all three human pathogen *Yersinia* species, a 9.5-kpb plasmid (pPCP1) and a 100 to 110-kbp plasmid (pMT1), both unique to *Y. pestis* (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Brooks, et al., 2004). The numbers in the plasmids' names indicate the strain of *Y. pestis*, in this case from a strain called KIM, and different strains have different numbers (Perry & Fetherston, 1997).

The pCD1 (calcium dependence) plasmid, known as pCad or pYV in some strains as well as the enteropathogenic yersiniae, contains the genes needed to produce the type III secretion system – *yop* (yersinia outer membrane protein), *ysc* (yersinia secretion) and *lcr* (low-calcium response) genes – which allows the bacterium to inject toxic substances into mammalian cells. Many of these genes (particularly *yop* and *lcr* genes) are part of a multicomponent virulence regulon, properly known as the low-calcium response (LCR), which consists of a set of regulatory genes, which regulate expression of the virulence genes in response to temperature, calcium and nucleotides such as ATP, and 12 regulated virulence genes, 11 of which encode a set of proteins called Yops and the $12^{th}$ being one of the *lcr* genes, the gene for LcrV/V antigen production (*lcrV*). Some of the regulatory genes include *lcrF*, which encodes a transcriptional activator that induces Lcr and Yop expression in response to the mammalian host's temperature, and *lrcK*, which functions in Yop secretion. There is also *lcrH*, which downregulates transcription of its own operon (which includes *lcrV*) and of Yop operons in response to both calcium and nucleotide presence at $37^{o}$C and *lcrQ*, which is a negative regulator of LcrF-mediated induction and requires the aid of YopD. Finally, there are also *lcrE* (whose product is called LcrE but also YopN) and *lcrG*, the products of which, according to an accepted model, work together with TyeA to block the secretion channels due to the presence of calcium in the medium and lack of cell contact with a host cell. Some of the adhesin and invasin genes in this plasmid, while active in the other two *Yersinia* species, are inactive in *Y. pestis* (e.g. *yadA* and

*inv*) (Price, et al., 1991; Perry & Fetherston, 1997; Nilles, et al., 1998; Fields & Straley, 1999; Salyers & Whitt, 2002).

The LCR genes are maximally expressed at 37$^{o}$C (mediated by LcrF) in the absence of calcium and nucleotides but, while secretion of Yops also occurs in the enteropathogenic yersiniae, this does not occur in *Y. pestis* without contact with a eukaryotic cell, as Yops are promptly degraded by Pla after translation. This maximal expression is accompanied by a cessation of bacterial growth called restriction. In the presence of millimolar concentrations of calcium or nucleotides, expression at 37$^{o}$C is partial, instead of maximal, but while expression of Yops occurs, secretion does. This partial expression also means that there is no restriction of bacterial growth (Price, et al., 1991; Nilles, et al., 1998; Brubaker, 2003). It has been proposed that the *in vitro* phenomenon of expression of LCR genes due to a calcium-free environment occurs because the calcium-free medium mimics an *in vivo* signal that occurs when *Y. pestis* enters into cell-to-cell contact with a eukaryotic cell, which is why Yop expression and secretion is found occurring in bacteria in contact with eukaryotic cells even in a medium with calcium present (Fields & Straley, 1999).

The pPCP1 (pesticin, coagulase, plasminogen activator) plasmid carries a gene encoding a plasminogen activator protease (*pla*) which, asides from activating plasminogen, cleaves complement component C3, degrades Yops promptly after translation and fibrin, adheres to the extracellular matrix component laminin and also appears to be important for dissemination of the pathogen fairly early in infection due to flea bite by preventing chemotaxis of PMNs to the site of infection. It also appears to be essential for full virulence in bubonic plague but not in septicaemic plague. This plasmid also contains genes for the bacteriocin pesticin (*pst*) and a pesticin immunity protein (*pim*) (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Brubaker, 2003; Josko, 2004; Prentice & Rahalison, 2007).

Finally, the plasmid pMT1 (murine toxin), also known as pFra in some strains, carries genes for the production of the *Y. pestis* capsule-like F1 antigen – *caf* (capsular antigen fraction) genes – as well as 74 other genes, including a gene (*ymt*) encoding for a protein once called *Yersinia* murine toxin, but now known as phospholipase D (PlD) which, despite its previous name, does not actually have a role in pathogenesis in

mammalian hosts but is, instead, important in colonization of the flea gut, especially as it is expressed more highly at the flea's body temperature than a mammal's (Perry & Fetherston, 1997; Salyers & Whitt, 2002; Rollins, et al., 2003). Phospholipase D appears to be needed to protect the bacterium from antibacterial factors in the flea midgut (Prentice & Rahalison, 2007). F1 antigen is described in greater detail in its own section further ahead.

The two extra plasmids, pPCP1 and pMT1, might explain part of the higher virulence of *Y. pestis* compared to the other yersiniae but the inactivation of some genes might also play a role in this (Perry & Fetherston, 1997; Salyers & Whitt, 2002). Furthermore, transcriptomic analysis and comparison of the genes common between *Y. pestis* and *Y. pseudotuberculosis* have revealed that 91% of these common genes are more highly expressed in *Y. pestis* than *Y. pseudotuberculosis,* including the chromosome-borne High Pathogenicity Island (HPI) which encodes yersiniabactin, a siderophore involved in iron acquisition, the plasmid pCD1/pYV which encodes the type III secretion system (TTSS) and the outer membrane protein Ail. All of these encode for important virulence factors, therefore the higher virulence in *Y. pestis* compared to the other yersiniae might not only be due to gain of some genes and loss of others but also due to differences in gene expression (Chauvaux, et al., 2010).

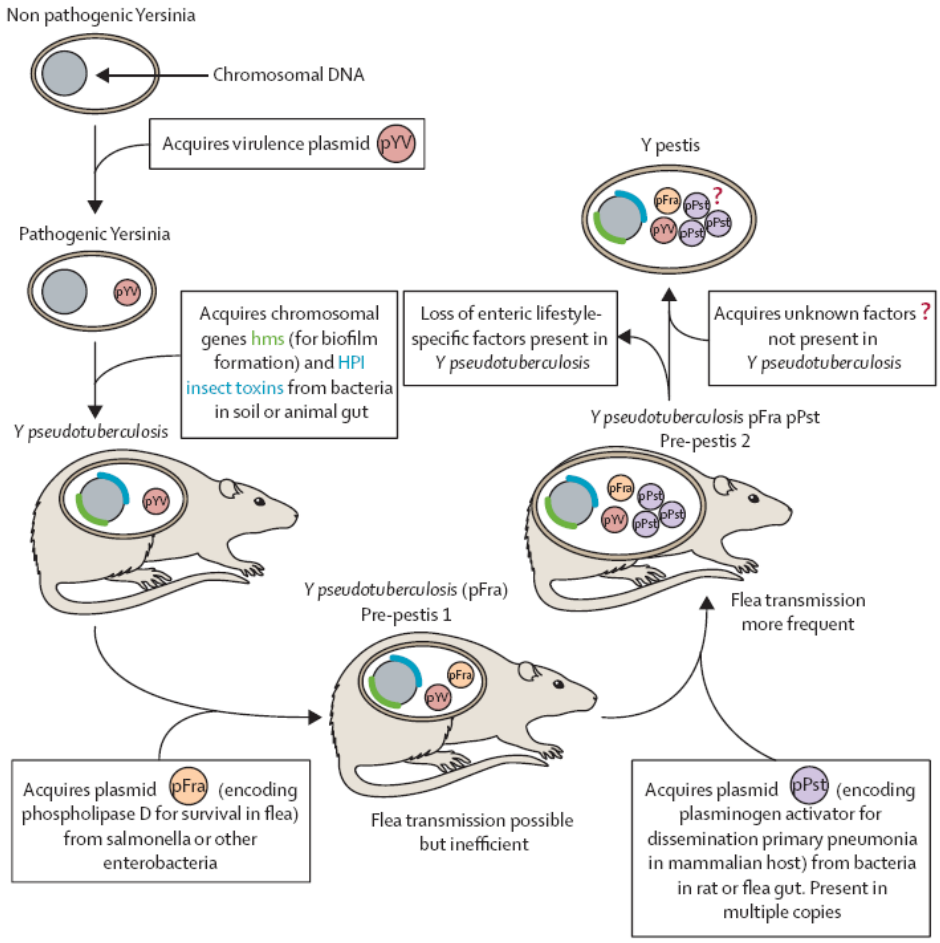


**Figure 1.8 –** Model of pathogenic *Yersinia* species evolution based on genome data. Non-pathogenic yersinae first gain the virulence plasmid pYV (pCD1) to form the predecessor of pathogenic yersinae. *Y. pseudotuberculosis* gains the ability to parasitize insects by, in part, building on pre-existing biofilm capacity and later evolves into *Y. pestis* by acquiring the plasmids pPst (pPCP1) and pFra (pMT1 and suffering some genetic loss **(Prentice & Rahalison, 2007)**.

The *Yersinia* species are capable of injecting their virulence factors directly into the cytoplasm of host cells by way of a formation of tubular structures in the membrane known as a Type III secretion mechanism, a mechanism comparable to a 'syringe and needle' system (Lee & Schneewind, 1999). The core of the secretion system's structure can assemble in the absence of a eukaryotic cell since the genes are maximally expressed at 37$^{o}$C. However, the translocated proteins are not expelled from the bacterium unless contact with a eukaryotic cell is made and the full injection structure is assembled. Until the protein gate of the secretion system's channel makes contact with the low-calcium environment of the eukaryotic cell's cytoplasm, it remains closed, not allowing secretion. Once it opens, however, secretion, in an ATP-dependent manner,

may occur and the secreted proteins have a variety of effects on the eukaryotic cell, all working to kill it (Salyers & Whitt, 2002).

### 1.2.3. Evasion Strategies

*Y. pestis* can stimulate overexpression of inhibitory molecules (such as IL-10). Its ability to survive inside macrophages during early infection allows it to escape recognition by B-cells and in order to preserve itself it can inhibit, among others, MHC and CD1 expression, cytokine release, proteosomes and apoptosis (Prentice & Rahalison, 2007; Playfair & Bancroft, 2008). Another characteristic which helps the bacterium survive is to diminish the host response to LPS by, at 37$^{o}$C, remodelling the hexa-acylated lipid A structure of its LPS to be less acylated (tetra-acylated) and less toxic (500-fold less toxic). Tetra-acylated LPS is not only less stimulatory to TLR4 (the receptor which recognizes LPS) but also acts as an antagonist for the stimulatory hexa-acylated LPS, which helps prevent activation of macrophages, secretion of proinflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8) and activation and maturation of dendritic cells which are required for the induction of adaptive immunity (Prentice & Rahalison, 2007; Li & Yang, 2008).

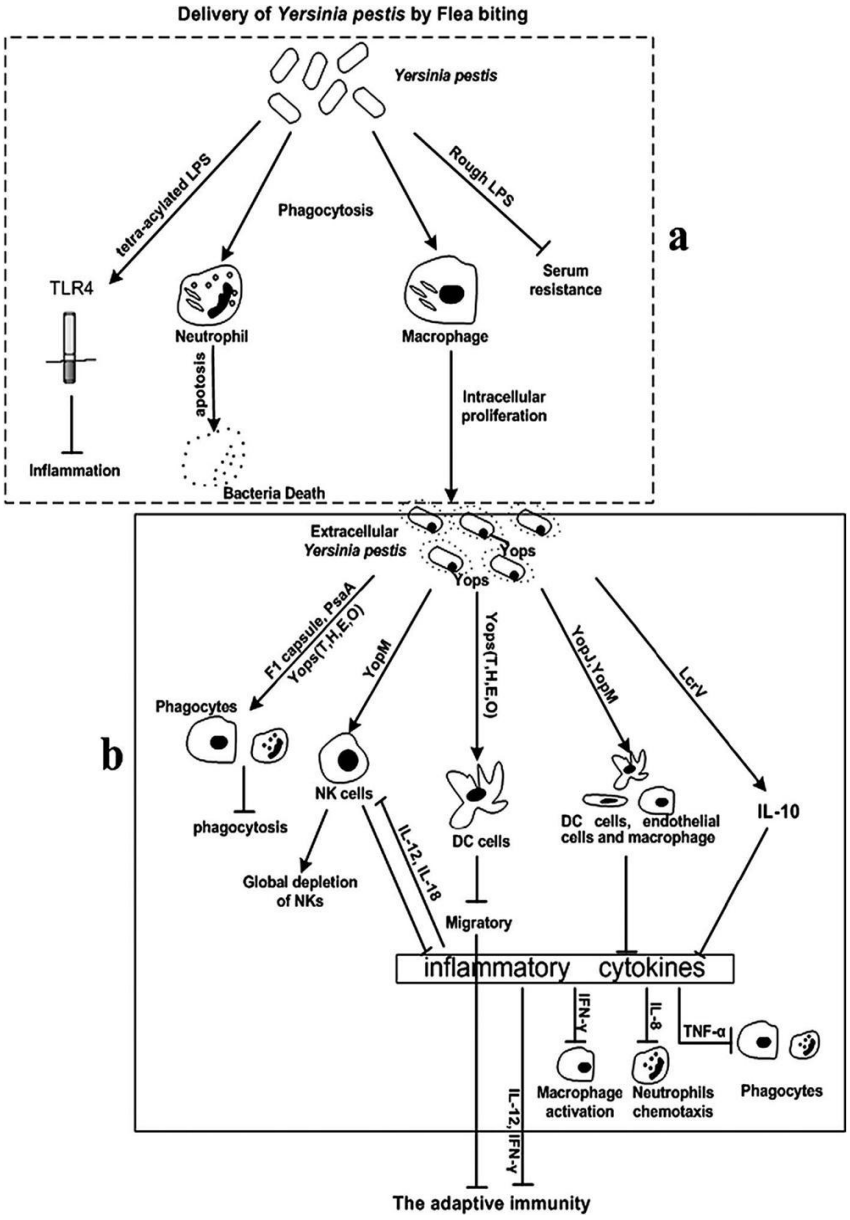


**Figure 1.9 –** Some of *Y. pestis'* defence mechanisms against host innate immunity. (a) Defence mechanisms at the early stage of infection; (b) Defence mechanisms after the release of *Y. pestis* from macrophages. Image slightly altered from source to better fit current understanding **(Li & Yang, 2008)**.

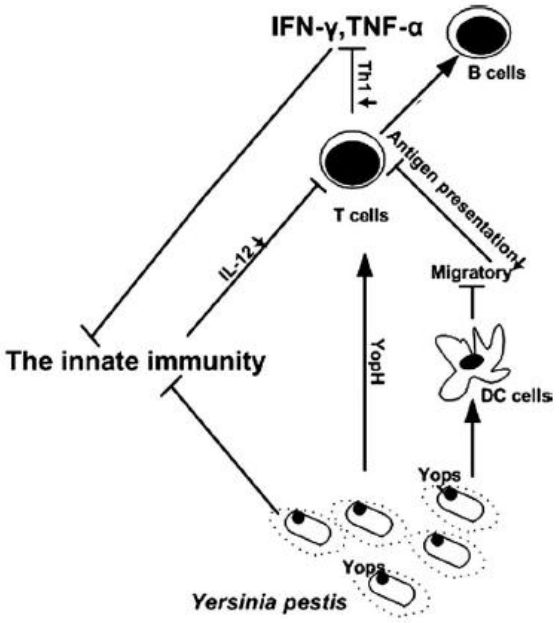


**Figure 1.10 –** Defense mechanisms of *Y. pestis* against the host's adaptive immunity and the link between innate immunity and adaptive immunity (Li & Yang, 2008).

1.2.4. Fraction 1 (F1) Antigen or Caf1

The 15.5-17.5 kDa capsular protein Fraction 1 (F1), also known as Caf1 and encoded by the *caf1* gene in the pMT1 plasmid, which exists only in *Y. pestis,* forms a capsule-like structure and has antiphagocytic properties (Miller, et al., 1998; Salyers & Whitt, 2002). In order to assemble its fibrillar structures, it requires Caf1M, a chaperone that allows export of F1 subunit to the surface, and Caf1A, an usher that anchors F1 into the outer membrane. Another protein, Caf1R, is responsible for regulating the expression of these proteins in response to temperature. The *caf1*, *caf1R*, *caf1M* and *caf1A* genes are organised into the *caf* operon, which is usually expressed at 37$^{o}$C (Miller, et al., 1998; Sodhi, et al., 2004).

Research by Du and coworkers, using J774 mouse macrophages, showed that the chaperone Caf1M is essential for surface expression of F1 antigen, that F1 antigen reduces bacterial binding to macrophages and confirmed that F1 increases the ability of *Y. pestis* to resist phagocytosis, probably by preventing adhesion-receptor interactions (Du, et al., 2002).

Miller and colleagues' findings revealed that F1 can exist as high-molecular-weight forms and that F1 can naturally reassemble into multimers in the size range of 80-200,000 kDa after denaturation (Miller, et al., 1998). Later, they used mass spectrometry to examine subunit interactions in F1 antigen. The results from mass spectrometry suggested that there likely are no F1 monomers *in vivo* and they deduced that the formation of the macromolecular F1 antigen structure involves seven F1 subunits in each helical turn with a solvent-filled channel (Tito, et al., 2001).

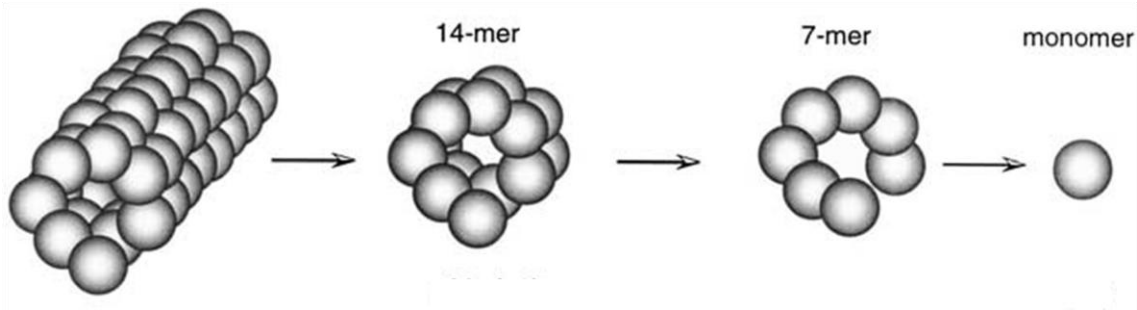


**Figure 1.11 –** Schematic representation of the dissociation of F1 antigen macromolecular structure proposed from mass spectrometer results **(Tito, et al., 2001)**.

Zavialov and colleagues' study on F1 antigen fiber formation ended up contradicting Tito *et al.*'s helical model of F1 assembly. They had also previously suggested that the helices Tito *et al.* had observed might have actually been circles of Caf1 monomers attached by donor strand exchange (DSE). In their study, they gave direct evidence for DSE assembly and uncovered the structures of the Caf1M:Caf1 preassembly complex and of the Caf1M:Caf1:Caf1 structure, the smallest possible F1 fiber (Zavialov, et al., 2003).

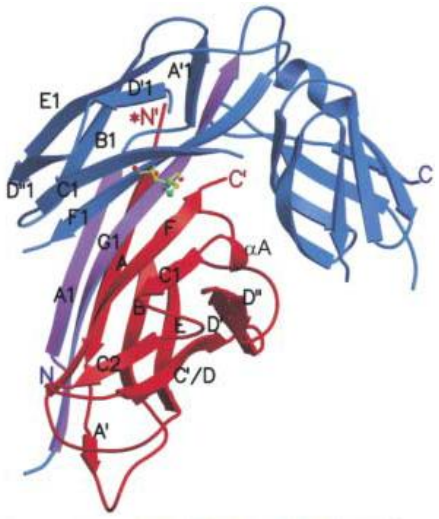


**Figure 1.12 –** Diagram of the Caf1M:Caf1 complex. Caf1M is blue with edge strands $G_1$ and $A_1$ in violet; Caf1 is red and the *N' label indicates that the N-terminal donor sequence of Caf1 was replaced by a His-tag **(Zavialov, et al., 2003)**.

Together with genetic data, this made them propose a model where assembly is driven by folding energy being preserved by the chaperone. According to this model, Caf1M traps the chaperone bound Caf1 subunit in a molten globule-like shape, stopping the folding process. DSE occurs when one of the two strands (G1 in Figure 1.12) that the chaperone has in the accepting cleft of Caf1 is exchanged for the donor strand of a neighbouring Caf1 and the other chaperone strand (A1 in Figure 1.12) is removed without a replacement (Zavialov, et al., 2003).

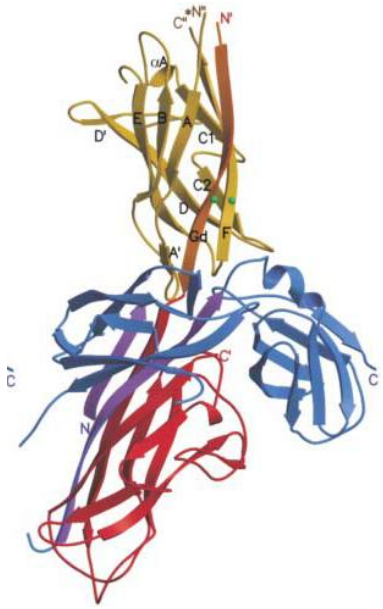


**Figure 1.13 –** Diagram of the Caf1M:Caf1:Caf1 complex. Caf1M is blue with edge strands $G_1$ and $A_1$ in violet; Chaperone bound Caf1 subunit is red; Caf1 subunit corresponding to the tip of a growing fiber is yellow, the *N' label indicates that the N-terminal sequence of Caf1 is disordered and the donor strand ($G_D$) is orange **(Zavialov, et al., 2003)**.

DSE is accompanied by the finishing of the folding of the original chaperone-bound Caf1, releasing free energy that drives assembly onward, and by Caf1M binding to the new subunit and stopping its folding much like the original Caf1 (Zavialov, et al., 2003). This gave rise to the linear fibre model of F1 antigen assembled structure.

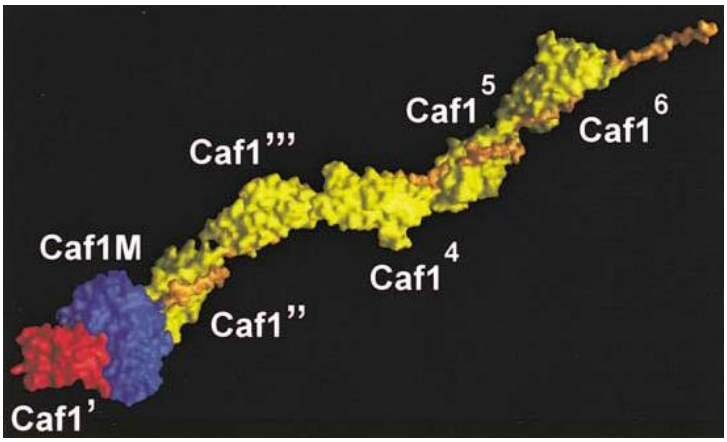


**Figure 1.14 –** Molecular surface rendering of a model for a F1 fiber generated by assuming the same orientation between successive subunits as seen for Caf1' and Caf1''. Caf1M is blue; Chaperone bound Caf1' is red; Remaining Caf1 subunits are yellow and the $G_D$ β strand is orange **(Zavialov, et al., 2003)**.

Sharma and colleagues studied the effects of *in vitro* rF1 stimulation of Balb/c mouse peritoneal macrophages and verified that rF1 activated macrophages, resulting in upregulation of TLR5 (but no change of TLR2 or TLR4 expression), increased production of nitric oxide (NO), TNF-α, IL-1, IFN-γ and IFN-γ chemokines KC, IP-10, MIP-1α, MIP-1β, MCP-1 and RANTES (Sodhi, et al., 2004). They later focused on the role of p42/44 (ERK1/ERK2) MAPK-mediated signal transduction in the same kind of murine peritoneal macrophages. They noticed that rF1-induced activation of p42/44 MAPK correlated with functional activation of macrophages, including actin rearrangement, production of NO, TNF-α and IL-1β but not phosphorylation of p38 MAPK. They also concluded that rF1-induced signal transduction process in macrophages is mediated through protein kinase C, tyrosine kinases and phosphoinositol-3-kinase (PI3-K). Furthermore, rF1 also activated JNK MAPK (Sharma, et al., 2005).

Caf1A is essential for assembly as it dissociates the Caf1 subunit from any further Caf1M:Caf1 complexes that bind to it and drives the subunit to the emerging F1 fiber, which later allows it to secrete to the extracellular medium by an unknown mechanism (Dubnovitsky, et al., 2010).

### 1.2.5. LcrV or Virulence (V) Antigen

LcrV, also known as V antigen, is a 327-residue, 31-39 kDa soluble protein encoded by the *lcrV* gene in the pCD1/pYV plasmid common in all three human-pathogenic *Yersinia* species and is a multifunctional protein with a vital role in regulating the translocation of Yops and having anti-host functions of its own, therefore being required for virulence, and also being an active and passive mediator of disease resistance (Price, et al., 1991; Schmidt, et al., 1999; Fields, et al., 1999; Derewenda, et al., 2004; Sodhi, et al., 2005). Derewenda *et al.* managed to crystallize *Yersinia pestis* LcrV and determine its structure (Derewenda, et al., 2004).

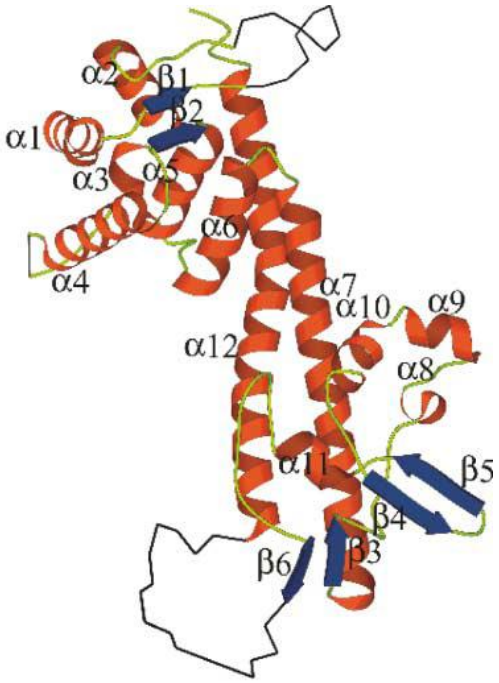


**Figure 1.15 –** Tertiary Structure of *Y. pestis* LcrV with α-helices and β-strands coloured orange and blue, respectively **(Derewenda, et al., 2004)**.

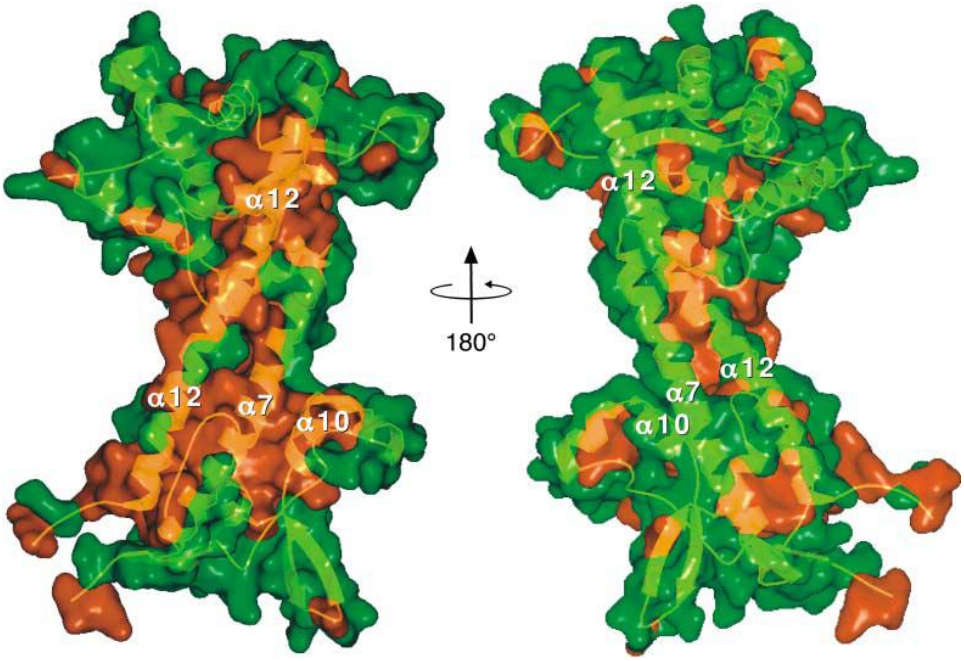


**Figure 1.16 –** Front and back views of the surface of LcrV. Aminoacid residues in brown are homologous to *P. aeruginosa* PcrV **(Derewenda, et al., 2004)**.

*Y. pestis* LcrV has been shown to be secreted into the extracellular medium in low-calcium environments, at 37$^{o}$C, regardless of cell contact, probably when it dissociates from the Ysc secretion apparatus, but only in extracellular bacteria. This secretion might occur either directly by Ysc or perhaps if the Yop-targeting complex is a dynamic structure, assembling and disassembling, causing release of LcrV from the complex into the medium. It is also secreted into host cells independent of the type III secretion system or the pCD1 plasmid but this also only happens if the bacterium is in an extracellular environment. LcrV also regulates the opening of the type III secretion channel (the injectisome, composed of a basal body embedded in the two bacterial membranes and an external needle protruding from the bacterial surface), being found present on the surface of the bacterium as the tip of the needle, bound to YscF (a component subunit of the needle) below it, before cell contact when YopB and YopD bind to the tip. It is also involved in translocating the Yops into the eukaryotic cell (Nilles, et al., 1998; Fields & Straley, 1999; Fields, et al., 1999; Pettersson, et al., 1999; Salyers & Whitt, 2002; Mueller, et al., 2005). Broz and colleagues determined what the molecular architecture of *Y. enterocolitica* LcrV injectisome tip complex would look like and that it is formed by three to five LcrV monomers, with a pentamer being the best fit for an atomic structure of this complex (Broz, et al., 2007). Caroline *et al.* showed that LcrV likely needs to denature in order to pass through the injectisome and reach the tip where it refolds into a doughnut-shaped oligomer, which could be either a tetramer or a pentamer, and this refolding might also require that LcrV is first denatured before refolding can occur (Caroline, et al., 2008).

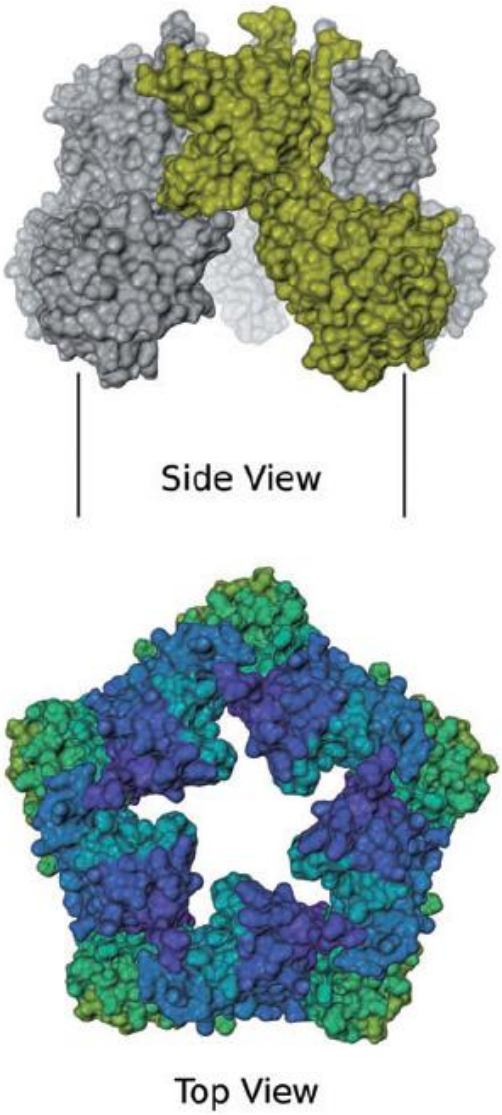


**Figure 1.17 –** Side and top view of the pentameric LcrV tip complex model at the tip of the injectisome needle. In the side view, a single monomer is highlighted in yellow, and in the top view, a colour gradient was applied for aesthetic reasons **(Broz, et al., 2007)**.

Within the bacteria, it appears to also be involved in the growth restriction seen in the low-calcium response as well as in upregulation of Yop and LcrH expression (Price, et al., 1991) by binding to the cytoplasmic, negative regulator, Ysc gate protein LcrG (Nilles, et al., 1998; Fields, et al., 1999).

1.2.5.1. Immunomodulatory Activity of V antigen

One of the reasons this protein is highly researched is that it possesses immunosuppressive ability. *Y. pestis* LcrV has been shown to suppress production of the cytokines TNF-α and IFN-γ in mice (Leary, et al., 1995). Schmidt *et al.* used a recombinant polyhistidine LcrV derived from *Y. enterocolitica* O8 to show that LcrV suppressed TNF-α in Balb/c mice, not by direct effect on macrophages, but with the help of activated T-cells and it does this, not with LcrV-induced IL-10 production, but with an LcrV-induced soluble factor produced by T-cells (without requiring cell-to-cell contact between macrophages and T-cells). Using anti-IL-10 antibodies did not stop or diminish TNF suppression. The resulting suppression appears to occur due to either decrease of transcription rate of TNF-α or increase of TNF-α mRNA degradation (Schmidt, et al., 1999).

Some clues as to how LcrV modulates innate immune responses have been provided by studies by Sing and colleagues. Their results appear to contradict previous findings by showing that IL-10 is not only induced in response to *Y. enterocolitica* O8 rV antigen stimulation in both *in vitro* models of murine peritoneal macrophages (from C3H/HeJ mice) and human macrophages (Mono Mac 6) but that IL-10 is indeed required for TNF-α suppression and T-cells were not used in these *in vitro* models. However, they did not actually disprove that an alternative mechanism of IL-10-independent TNF-α suppression through the rV-dependent secretion of an unidentified soluble factor from T-cells does not occur. They further showed that TLR4 is not involved in this process by stimulating mouse cells with a non-functional TLR4 (C3H/HeJ, Balb/c LPSd) and without TLR4 (C57BL/10ScCR) with V antigen, which still resulted in TNF-α suppression (Sing, et al., 2002). Sing *et al.* later demonstrated that *Y. enterocolitica* O8 rLcrV signals IL-10 production in a CD14-TLR2-dependent manner in both murine (peritoneal macrophages of different genetic backgrounds: C57BL/6 wild-type, C3H/HeJ, C57BL/10ScCR and Balb/c LPSd) and human cells (CD14-TLR2-cotransfected HEK 293), showing that both TLR2 and CD14 are necessary for IL-10 induction and identifying a new ligand specificity of TLR2 (Sing, et al., 2002).

Later on, Sharma and colleagues demonstrated that, in Balb/c mice peritoneal macrophages, LcrV innate immune recognition involves TLR2 as well as TLR6, but not TLR1 and that it results in the inhibition of transcription of some cytokines and chemokines (Sharma, et al., 2004; Sharma, et al., 2005; Sodhi, et al., 2005).

Reithmeier-Rost and his colleagues (from Sing's group) investigated whether *Y. enterocolitica* O8 rLcrV had the same effects they had observed in wild-type C57BL/6 mouse peritoneal macrophages in the human monocytic cell line Mono Mac 6. They verified once again that TNF-α secretion was suppressed and IL-10 expression was induced in response to rLcrV but they expanded these findings by stimulating the human and the murine cells and collecting and analysing samples during a 24 hour period. They observed that rLcrV induced TNF-α secretion, which peaked at 3h and subsequently decreased until reaching baseline levels past 24h in the human cells, and they found that rLcrV-induced IL-10 secretion was detected after 6h and kept increasing, reaching its peak after 24h. In contrast, in the murine cells they found that both TNF-α and IL-10 secretion rose almost in tandem in the first 6h, after which IL-10 levels started decreasing while TNF-α levels continued rising. IL-10 secretion was also confirmed to be induced in a TLR2/CD14-dependent manner following rLcrV stimulation in human Mono Mac 6 cells, thereby confirming that this dependence applied to more than one type of human cells. Other of their findings include rLcrV (TLR2 agonist) inducing auto-, homo- and heterotolerance, that IL-10 is important for rLcrV-induced tolerance, that rLcrV induces IL-10 secretion at long range, without requiring cell-to-cell contact and that rLcrV does not influence TLR2 or TLR4 expression in Mono Mac 6 cells (Reithmeier-Rost, et al., 2004).

Abramov and colleagues' study on the specificity and avidity of LcrV-TLR2 interaction resulted in the discovery that *Y. pestis* LcrV can bind not only to TLR2/CD14, therefore resulting in the secretion of IL-10 and suppression of TNF-α in a TLR-2/CD14-dependent manner, but can also bind to human (but not mouse) INF-γ, so long as the cytokine is also bound to its receptor, creating an LcrV/hIFNγ-hINFγR complex, resulting in TLR2/CD14-independent induction of IL-10 secretion and suppression of TNF-α (Abramov, et al., 2007). These findings appear to contradict some of Sing *et al.*'s findings that LcrV-induced IL-10 secretion is entirely TLR2/CD14-

dependent in human cells but this is possibly because they used *Y. enterocolitica* rLcrV, rather than *Y. pestis*, and the difference of amino acids between *Y. enterocolitica* LcrV from *Y. pseudotuberculosis* and *Y. pestis* is noted in this study, with *Y. enterocolitica* LcrV having an insertion of 9 amino acid residues that results in a slight change in the folded protein structure (Sing, et al., 2002; Reithmeier-Rost, et al., 2004; Abramov, et al., 2007). Gendrin and colleagues expanded upon Abramov *et al.*'s findings slightly by proving that, *in vitro* with purified *Y. pestis* rLcrV, IFN-γ and, sometimes, IFN-γR, rLcrV does actually bind to free hIFN-γ and with greater affinity, not just to the hIFN-γ/IFN-γR complex (Gendrin, et al., 2010).

Reithmeier-Rost and colleagues (including Sing), wanting to verify whether *Y. pestis* LcrV acted the same way as *Y. enterocolitica* O8 LcrV inducing IL-10 production in a TLR2/CD14-dependent manner, infected cells of certain types and species with either *Y. pestis* LcrV or *Y. enterocolitica* O8 LcrV. They found that, compared to *Y. enterocoltica*, *Y. pestis* LcrV resulted in a dramatically weaker TLR2/CD14-mediated response and in very low levels of IL-10 and TNF-α secretion. Other findings of theirs made them conclude that the presence of TLR2 does not change the final outcome of *Y. pestis* infection of mice and that since *Y. pestis* LcrV/TLR2 interaction is relatively weak, that TLR2-dependent IL-10 induction by LcrV does not contribute to virulence of *Y. pestis* (Reithmeier-Rost, et al., 2007).

## 1.3. Aims

The aims of this study were to determine the effects of *Y. pestis*-derived F1 antigen and V antigen on certain aspects of the human innate immune system. Based on the current literature, the hypothesis was that *Y. pestis* virulence factors are able to modulate innate immune responses. To this end, human cells from the monocyte/macrophage line were stimulated with either of these antigens in order to determine whether they affected TLR expression, phosphorylation of IκB (and therefore activation of NF-κB) and secretion of cytokines involved in the $T_H1$ and $T_H2$ response over a period of 24 hours. This study also sought to determine whether these antigens had any effect on tolerance by pre- or post-treating with F1 or V cells stimulated with agonists of different TLRs. Finally, the intracellular trafficking and targeting of F1 and V antigen was studied in response to different TLR agonists.

# *Chapter 2 - Materials and Methods*

## 2.1. Antibodies

**Table 2.1 –** Primary and secondary antibodies used in this study. Secondary antibodies were labelled with either a fluorescent fluorochrome (Fluorescein isothiocyanate {FITC}, Cyanine 5 {Cy5} and Tetramethyl rhodamine iso-thiocyanate {TRITC}) or horse radish peroxidase (HRP) for enhanced chemiluminescence (ECL).

| Primary Antibodies | Species | Company |
|---|---|---|
| Anti-F1 antigen [YPF19] | Mouse | Abcam |
| Anti-LcrV [Ab983] | Mouse | Abcam |
| Anti-TLR1 | Mouse | Hycult Biotechnology |
| Anti-TLR2 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR3 | Rabbit | Santa Cruz Biotechnology, Inc. |
| Anti-TLR4 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR5 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR6 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR7 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR8 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-TLR9 | Goat | Santa Cruz Biotechnology, Inc. |
| Anti-CD36 | Rabbit | Hycult Biotechnology |
| Anti-Phosho-IκB-α (Ser32) | Rabbit | Cell Signalling Technology, Inc. |
| Anti-GM130 [sc-55591] | Mouse | Santa Cruz Biotechnology, Inc. |
| Anti-EEA1 | Mouse | Santa Cruz Biotechnology, Inc. |
| Anti-MyD88 [sc-11356] | Rabbit | Santa Cruz Biotechnology, Inc. |

| Secondary Antibodies | Label | Company |
|---|---|---|
| Goat anti-mouse | FITC | DAKO Cytomation |
| Rabbit anti-goat | FITC | DAKO Cytomation |
| Swine anti-rabbit | FITC | DAKO Cytomation |
| Donkey anti-goat | Cy5 | Jackson ImmunoResearch Laboratories |
| Rabbit anti-mouse | TRITC | DAKO Cytomation |
| Rabbit anti-mouse | HRP | DAKO Cytomation |
| Swine anti-rabbit | HRP | DAKO Cytomation |

## 2.2. Cell lines

### 2.2.1. Tissue culture

To ensure sterile conditions during tissue culture a Microflow Advanced Biosafety Class II laminar flow hood was used. Plasticware, glassware and solutions were autoclaved for tissue culture. The hood and equipment was sterilized with 1% aqueous Virkon (Antec International). Cells were incubated at 37˚C with 5% $CO_2$ humidified atmosphere.

### 2.2.2. Mono Mac 6 cells

Mono Mac 6 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmBH, the German Collection of Microorganisms and Cell Cultures. The Mono Mac 6 cell line is a monocytic cell line that was established from the peripheral blood of a 64-year-old man with acute monocytic leukemia (AML FAB M5). It is currently the only mature human monocytic cell line available (Ziegler-Heitbrock, et al., 1988; Ziegler-Heitbrock, 2010).

Mono Mac 6 cells were seeded at a density of 2 x $10^5$/ml in 2 ml volumes in 24-well-plates and they are cultured at 37°C in a humidified 5% CO 2 atmosphere for 3-4 days before being used for experiments or before being pooled and reseeded again. Mono Mac 6 cells were cultured in 24-well plates in RPMI medium + GlutaMAX (GIBCO) supplemented with 10% foetal calf serum (FCS {Biosera}), 10 ml of non-essential amino acids (NEAA) (Invitrogen), as well as OPI media supplement 5 ml for 1 ltr (Sigma), which contains oxalacetic acid, sodium pyruvate and insulin.

### 2.2.3. Cryogenic preservation

Cryogenic preservation (<-100°C) was used to create and maintain reserve cell lines. Cell lines were placed in a -80°C freezer for 24h and then into liquid nitrogen for long-term storage. This step down in temperature allows cells to adjust to the liquid nitrogen.

Cells were washed and then re-suspended in 10 ml of their growth media by pipetting for semi-adhesive cells. Cells were placed in a 15 ml tube (Nunc) and spun at 12000 RPM for 5 minutes. The supernatant was aspirated off and the pellet re-suspended in 1500 µl of freezing medium (10% (v/v) dimethyl sulfoxide (DMSO) in FCS). Cells in freezing medium were quickly placed in cryotubes (Nunc) and then into the -80°C freezer for 24 hours. After 24 hours the cryotubes were removed from the -80°C freezer and placed in liquid nitrogen (-196°C).

## 2.3. V antigen

### 2.3.1. Recombinant LcrV plasmid (pVG110)

The recombinant LcrV plasmid (pVG110) with a glutathione S-transferase (GST) tag used in this study was kindly provided by Prof. Richard Titball (Chemical and Biological Defense Establishment, Porton Down, Salisbury, UK) as previously described (Carr, et al., 2000). The plasmid was transformed into *E. coli* BL21 cells.

### 2.3.2. Fermentation of LcrV

*E. coli* BL21 cells containing the recombinant plasmid pVG110 was cultured in Luria broth containing 100 mg/ml ampicillin. Cultures were incubated with shaking (120 RPM) at 37ºC until an $A_{600}$ of 0.3 was achieved; cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 5 h and harvested by centrifugation at 6500 RPM for 30 min.

### 2.3.3. Purification of LcrV

Once the broths had been centrifuged, the resulting pellets were resuspended in 10 ml of phosphate buffered saline (PBS) and placed in 50 ml tubes, the centrifuge container was washed out with a further 10 ml PBS and this was also added to the 50 ml tube. The *E. coli* suspension was then frozen (-20ºC) and thawed 6 times. Tubes were vortexed vigorously between freezing and thawing. The tubes were then centrifuged at 2500 RPM in a 4ºC centrifuge for 60 minutes. The supernatant was placed in a sterile 50 ml tube and the resulting pellet was discarded. The supernatant was then purified using a glutathione sepharose (GST) purification column supplied by GE Healthcare.

### 2.3.4. Glutathione sepharose (GST) protein purification

GST tagged proteins can be purified from lysate using glutathione immobilized on a sepharose matrix via ligand specificity (figure 2.1). The GST tag has a high affinity

for glutathione (GSH). Elution of bound GST fusion proteins from the column is achieved under mild, non-denaturing conditions (Wilson & Walker, 2005).

Attachment of the ligand (glutathione) to the affinity phase occurs via a spacer. The spacer allows optimum ligand interaction with the GST tagged protein.

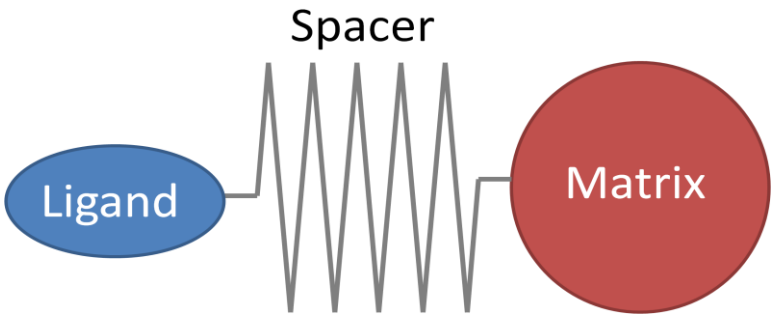


**Figure 2.1 –** Matrix/spacer/ligand interaction. The glutathione ligand (blue) is coupled via a 10-carbon spacer to highly cross-linked 6% agarose (purple). The spacer and ligand can be manipulated to suit the requirements of the separation.

To elute the GST-tagged protein from the column an enzyme is used to cleave the GST from the protein of interest and thus leave the relatively large GST tag (~26KDa) bound to the affinity matrix. PreScission™ Protease (GE Healthcare) was used in this study. This protease is highly specific and also has a GST tag so on elution it remains bound to the matrix eliminating need for extra purification thus increasing the resolution and yield of the eluate.

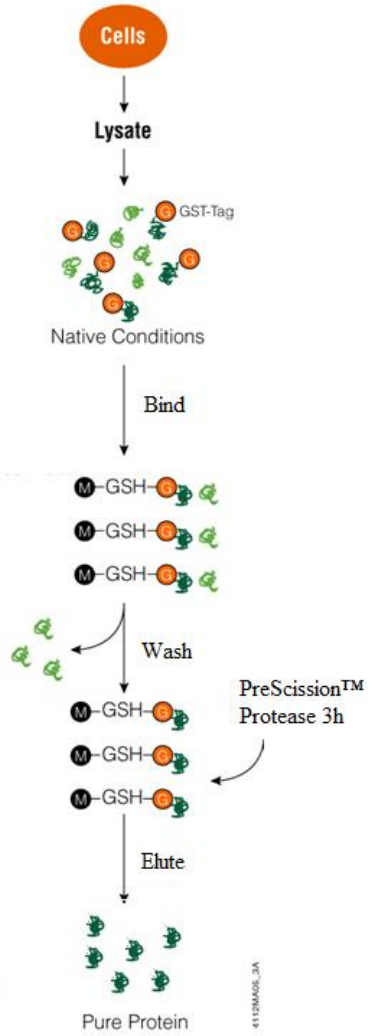


**Figure 2.2 –** GST-tagged protein purification. The GST tag (orange) has a high affinity for glutathione (GSH) and binds the protein of choice (dark green) to the affinity matrix. Washing removes unwanted lysate (light green). PreScission™ Protease (GE Healthcare) was used to cleave the GST tag from the protein leaving purified protein that does not require further purification. Image altered from source **(Promega Corporation, 2012)**.

### 2.3.5. LcrV-GST-tagged protein purification

The supernatant that was harvested from the LcrV fermentation was passed through a 2 ml glutathione sepharose (GST) purification column (GE Healthcare). The column was equilibrated by passing through 5 ml PBS/1% Triton. 2 ml crude V antigen was then passed in the column matrix and the column was sealed to terminate flow. The column was left to incubate at room temperature for 1hr. After incubation the column

was washed out with 6 ml PBS and the eluate was discarded. To the column 2 ml cleavage buffer (50mM Tris/150mM NaCl/1mM EDTA/1mM DTT/0.01% (w/v) Triton X-100) with 30 µl PreScission™ Protease (GE Healthcare) was then added and allowed to enter the matrix. The column was sealed to terminate flow. The column was then left to incubate for 3hr at room temperature. After the incubation, 5 ml cleavage buffer was used to wash out the column and the eluate collected in a sterile bottle.

### 2.3.6. Protein concentration

In order to concentrate the purified protein, 10 kDa cut off Centriprep centrifuge concentrators (Millipore) were utilised. Centriprep concentrators consist of two tubes, one housed in the other. The base of the internal tube has a permeable membrane that allows molecules through which are 10 kDa or smaller, retaining the protein in the external tube. During centrifugation the centrifugal force pushes anything that is ≤10 kDa through the membrane into the internal tube. The sample remains in the exterior tube whilst the waste runs through the membrane into the internal tube and is discarded.

Sterile 10 kDa cut off Centriprep concentrators (Millipore) were filled to the "fill line" with purified sample. The concentrators were then placed in the centrifuge and spun at 2500 RPM for 40 minutes at 4$^{o}$C. The content of the internal tube was discarded. The content of the exterior tube was placed back into the original elution and any precipitate re-suspended. This process was repeated until approximately 1-2ml of purified and concentrated protein remained. Sterile PBS was used to perform buffer exchange on the protein sample. PBS was filled to the "fill point" and the Centriprep spun at 2500 RPM for 40 minutes at 4$^{o}$C. This was performed three times with an end sample of approximately 1-2 ml. Protein concentration was determined by spectrophotometry as detailed further ahead (Section 2.3.9).

### 2.3.7. Endotoxin removal

Bacterial endotoxin contamination has proven a big problem in research, especially in the field of immunology. When expressing a protein in an *E. coli* strain,

for example, endotoxin from the *E. coli* can easily contaminate the purified product. Any cell stimulations performed with the contaminated protein will have a double effect of the protein itself and the contaminant, producing inconclusive data. To avoid this problem, endotoxin can be removed by ultra-filtration, ion exchange chromatography, two phase extraction or ligand specificity chromatography. In this study, the Profos EndoTrap® blue 10 (Hycult Biotechnology) kit was used to remove endotoxin from purified protein samples, this assay utilizes ligand specificity chromatography (Hycult® Biotech, 2011).

Profos EndoTrap® blue is an affinity matrix that is designed to remove bacterial endotoxins from aqueous solutions, even at low endotoxin concentrations. Pre-packed columns have the EndoTrap blue ligand covalently bound to beaded agarose. The EndoTrap blue ligand is highly specific for bacterial endotoxin and has very low non-specific binding, giving high sample yields free of endotoxin (Hycult® Biotech, 2011).

#### 2.3.7.1. Endotoxin removal protocol

Profos EndoTrap® blue 10 assay columns (Hycult Biotechnology) were firstly regenerated. Three times the column volume of regeneration buffer (Hycult Biotechnology) was passed through the column and the eluate discarded and this step was repeated. Three times the column volume of equilibration buffer (Hycult Biotechnology) was passed through the column, the eluate discarded and this step was repeated. A volume of sample equal to the column's volume was passed through it and the eluate was collected in sterile glassware. Three times the column volume of equilibration buffer was then passed through the column and collected in the same sterile glassware. The purified sample was re-concentrated in sterile Centriprep concentrators (Section 2.3.7) to a final volume of 1-2 mls.

### 2.3.8. Endotoxin detection

The HyCult LAL Chromogenic Endotoxin Quantitation Kit was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* amoebocyte lysate (LAL) assay. The LAL method for measuring endotoxin is based on

the interaction of endotoxins with the proenzyme Factor C found in circulating amoebocytes of the horseshoe crab *Limulus polyphemus*. The proteolytic activity of this proenzyme is activated in the presence of LPS. The Chromogenic *Limulus* amoebocyte lysate assay measures endotoxin levels by measuring the activity of this protease in the presence of a synthetic peptide substrate that releases p-nitroaniline (pNA) after proteolysis, producing a yellow color that can be measured by reading the absorbance at 405 nm (Hycult® Biotech, 2011).

To accurately measure endotoxin levels in a sample, the LAL assay uses an endotoxin standard of known concentration that is derived from *E. coli* strain O111:B4. This standard is provided with each kit and is used to create a standard curve. The endotoxin concentration is determined by extrapolating the absorbance of an unknown sample against this standard curve, similar to ELISA or total protein quantitation assays. Minimum concentration of LPS which can be measured is 0.01 EU/ml (1 EU = 100 pg LPS).

Following endotoxin removal using the Endotrap columns, all purified recombinant proteins were tested for the presence of endotoxin using the LAL assay. It was shown that there were no detectable endotoxin levels in the purified samples.

### 2.3.9. Determination of protein concentration

After the Endotrap step and concentrating the sample, a UV spectrophotometer was used to determine the concentration of the sample by use of the formula shown below. The theory behind this formula is that the aminoacid residues tyrosine and tryptophan within a protein show maximum absorption at a 280 nm wavelength. While the proportion of these residues within a protein do vary, and so do the extinction coefficients for the proteins, most proteins have an extinction coefficient within the range of 0.4-1.5. Following one of the Beer-Lambert law's equations (Absorbance = extinction coefficient x concentration of solution x length of solution) a fair approximation of the protein concentration of a solution in a quartz cuvette with 1 cm length can be calculated. However, there can often be contamination of nucleic acids which have an absorbance 10 times that of protein at this wavelength so even a small

amount of nucleic acids can greatly influence absorbance. The formula corrects for this possibility (up to 20% contamination with nucleic acids) by also measuring the sample's absorption at 260 nm wavelength, the maximum absorbance of nucleic acids, multiplying that by a ratio determined by early researchers and subtracting that value from the one obtained from the absorbance at 280 nm (Wilson & Walker, 2005; Boyer, 2006).

A UV spectrophotometer was used to determine the absorbance of the purified and concentrated V antigen solution at 260 nm and 280 nm. Then, concentration was calculated using the following formula:

$$[\text{Protein}]\ (\text{mg/ml}) = 1.55 \times A_{280} - 0.76 \times A_{260}$$

## 2.4. F1 antigen

### 2.4.1. Recombinant F1 plasmid pAH34L

The F1 plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6 that was used in this study was kindly provided by Prof. Richard Titball (Chemical and Biological Defense Establishment, Porton Down, Salisbury, UK) as previously described (Miller, et al., 1998). The plasmid was transformed into *E. coli* JM101 cells.

### 2.4.2. Fermentation of recombinant F1

E. coli JM101 containing plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6, was cultured in L-broth containing 10 mg/ml kanamycin. Cultures were incubated with shaking (120 RPM) at 37$^{o}$C until an $A_{600}$ of 0.3 was achieved; cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 5 h and harvested by centrifugation at 6500 RPM for 30 min.

### 2.4.3. Purification of F1 antigen

Once the broths had been centrifuged, the resulting pellets were resuspended in 10 ml of phosphate buffered saline (PBS) and placed in 50 ml tubes, the centrifuge container was washed out with a further 10 ml PBS and this was also added to the 50 ml tube. The *E. coli* suspension was then frozen (-20ºC) and thawed 6 times. Tubes were vortexed vigorously between freezing and thawing. The tubes were then centrifuged at 2500 RPM in a 4ºC centrifuge for 60 minutes. The supernatant was placed in a sterile 50 ml tube and the resulting pellet was discarded.

### 2.4.4. Preparation of F1 immunoaffinity columns

In order to prepare an affinity column for F1 antigen purification, a mouse monoclonal antibody against *Y. pestis* F1 antigen (Abcam, USA) was conjugated onto a

HiTrap column. The antibody (600 μg) was mixed with 800 μl of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl pH 8.3). A HiTrap luer adaptor was connected to the top of the HiTrap column and 1 ml of 1 mM HCl added to the top of the adaptor.

Following this, a syringe was used to gently wash out the isopropanol from inside the column with 6 ml of 1 mM HCl (at the rate of about 1 drop every 3 seconds). Immediately after this, the prepared antibody solution was injected onto the column, which was then sealed and left to stand for 4 hours at room temperature.

After the 4 hours, in order to deactivate the active sites on the column, 6 ml of Buffer A (0.5 M ethanolamine, 0.5M NaCl pH 8.3) were injected into the column, followed by 6 ml of Buffer B (0.1 M sodium acetate, 0.5 M NaCl pH4) and then another 6 ml of Buffer A again. The column was left to stand for 1 hour. At the end of the 1 hour period, the column was injected with 6 ml of Buffer B, then 6 ml of Buffer A and once more 6 ml of Buffer B. At the end of this, the column was injected with 2 ml of PBS pH 7 in order to adjust the pH of the column to a neutral pH. The column was ready to be used after this for the purification of the recombinant F1 proteins.

### 2.4.5. F1 recombinant protein purification

In order to purify the recombinant F1 protein, 1.5 mls from the supernatants from the L-broths that were centrifuged were injected into the column and the column was left to stand for about 2 minutes. The column was then injected with 5 ml of PBS, pH 7 in order to wash off any unbound proteins. The recombinant F1 proteins were eluted by with the adding of 4 ml of 0.1 % trifluoacetic acid pH 5 (TFA elution buffer) and the eluate was collected as it contained the F1 antigen. The procedure was then repeated from the beginning as long as there was more supernatant. Once all of the supernatant had been run through, the column was equilibrated with 5 ml of 1x PBS, pH 7, capped closed and stored.

Following the purification of F1 recombinant protein, the eluted proteins were concentrated, the endotoxin was removed and LAL assay was performed, as described above.

## 2.5. Cell stimulations

All stimulations were carried out in hybridoma Serum Free Medium (SFM, GIBCO) and incubated at 37°C in a 5% $CO_2$ humidified atmosphere. The use of SFM eliminates any effects that medium proteins may have on the experiment.

### 2.5.1. 24-well plate stimulation

Mono Mac 6 cells were stimulated in 24-well plates with a surface area of 1.9 $cm^2$ (Nunc). Sterile conditions were practiced throughout. The growth medium was aspirated off and the cells were washed with 1 ml SFM (GIBCO). 500 µl SFM was then added to each well. The cells were then either not stimulated or stimulated with different concentrations (5 µg. 50 µg or 100 µg) of V or F1 recombinant proteins, or with different TLR ligands such as: *E. coli* lipopolysaccharide (LPS) (100 ng), *S. aureus* lipoteichoic acid (LTA) (5 µg), single-stranded RNA (ssRNA) (5 µg) isolated from Coxsackievirus B3, Poly:I-C (20 µg) and CpG DNA (5 pmol). In addition, stimulations were performed where Mono Mac 6 cells were either pre- or post-treated with V or F1 antigen following stimulation with TLR ligands.

## 2.6. Immunofluorescence

Immunofluorescence is a technique whereby an antigen present within or on a cell can be detected using a specific antibody conjugated to a fluorophore (fluorescent molecule), with the amount of fluorescence emitted equating to the amount of antigen present. Fluorophores absorb light (energy) of a specific wavelength and re-emit energy of a different but specific wavelength. The most commonly used fluorophore is FITC (fluorescein isothiocyanate), which has an excitation wavelength of 495 nm (cyan) and an emission wavelength of 519nm (green). Another commonly used fluorophore is TRITC (tetramethylrhodamine isothiocyanate), which has an excitation wavelength of 547 nm (green) and an emission wavelength of 572 nm (yellow). There are two major types of immunofluorescence: primary (direct) and secondary (indirect) (Wilson & Walker, 2005; Rahman, 2006).

### 2.6.1. Principle of indirect immunofluorescence

Immunofluorescence is the labelling and visualisation of specific molecules with fluorescent monoclonal antibodies. Immunofluorescent labelling can be utilised to find the relative abundance and localisation of a chosen antigen.

Due to their specificity, antibodies, also known as immunoglobulins, are powerful analytical tools. Conjugated to a label of some kind, such as a radioactive isotope, an enzyme or a fluorochrome (also known as fluorophore), these antibodies allow us to qualitatively or quantitatively detect the antibody and therefore the protein for which the labelled antibody is specific to (which can be an antigen or even another antibody) (Wilson & Walker, 2005).

Typically they vary between two types: direct, where the labelled antibody is the primary antibody, that is, binds directly against the antigen of interest; and indirect, where the labelled antibody is the secondary antibody, which is the one which binds to the primary antibody, as opposed to binding directly to the antigen. The indirect technique is the most commonly used as this allows for: greater amplification of the signal with more than one secondary antibody being able to bind to a primary antibody;

each primary antibody does not have to be labelled individually as the secondary antibody will be specific for the species of the primary antibody, allowing the same secondary antibody to be used against different primary antibodies of the same species; loss of reactivity of the primary antibody as a result of labelling it does not occur (Wilson & Walker, 2005).

### 2.6.2. Principles of flow cytometry

In regards to flow cytometry, this technique allows us to quantify the intensity of fluorescence as well as quickly analyse many thousands of cells and even sort cells according to parameters specified by the user, such as fluorescence intensity and size of cell (Wilson & Walker, 2005).

The flow cytometer used in this study, a Fluorescence Activated Cell Sorter (FACS) can be divided into three main components: fluidics, optics and electronics or signal processing (Rahman, 2006).

The fluidics system is the part of the machine which forces the particles in the sample solution to pass through the optics system in single file through a process called hydrodynamic focusing (Rahman, 2006).

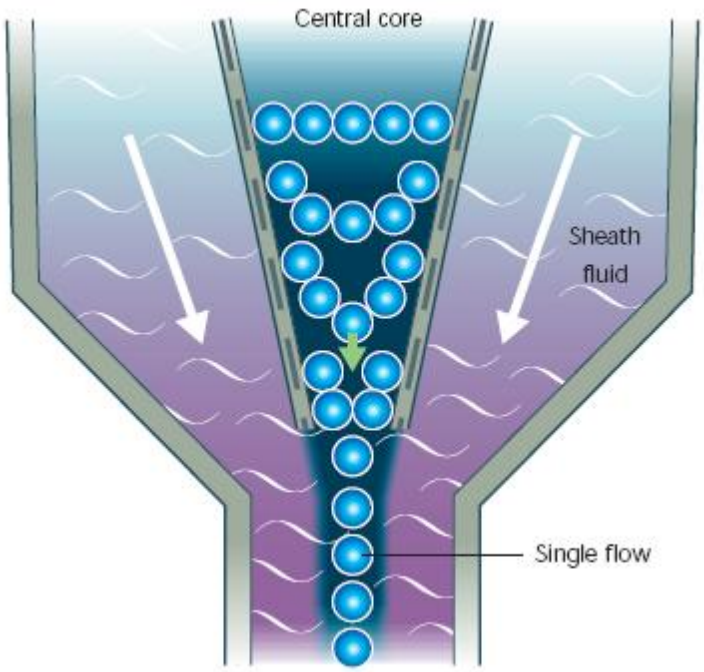


**Figure 2.3 –** Image showing how hydrodynamic focusing forces the sample fluid in the central core into a single stream of particles **(Rahman, 2006)**.

The optics system is the detection part of the machine where each particle passes through a beam of light (a laser in the case of the FACSCalibur™ machine used in this study) and detectors available pick up the resulting light emissions. The specificity of detection is controlled by optical filters which allow only certain wavelengths of light to pass through while absorbing the rest except in the case of dichroic mirrors/filters which redirect the rest of the light (Rahman, 2006).

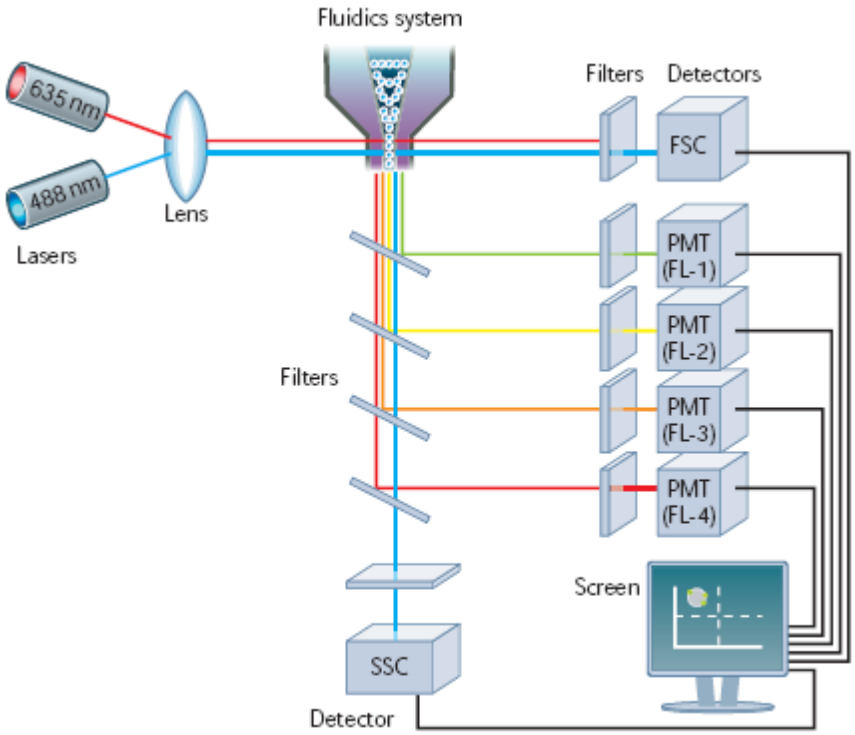


**Figure 2.4 –** A schematic of a typical flow cytometer setup (Rahman, 2006).

The electronics system, that is, the signal processing, was performed in this study with the aid of the Cell Quest software (BD Biosciences).

2.6.3. Pattern recognition receptor expression detection

Indirect immunofluorescence and flow cytometry were utilised in order to elucidate TLR receptor expression on Mono Mac 6 cells in response to V and F1 recombinant proteins.

To perform the indirect immunofluorescence assay, cell samples (1 x $10^6$ cells) in 1.5 ml Eppendorf tubes were washed (centrifuged at 1300 RPM for 2 minutes, re-suspended in 500 µl PBS/0.02% BSA {Bovine Serum Albumin [Sigma]}/0.02% $NaN_3$ and then centrifuged once again at 1300 RPM for 2 minutes) twice. The cells were then fixed by adding 300 µl of 4% paraformaldehyde (PFA {Sigma}) and left to incubate at room temperature for 10 minutes. Cells were then washed two times. The samples were then re-suspended in 200 µl PBS/0.02%BSA/0.02% $NaN_3$. To this, 2 µl of primary

antibody against the molecule of interest were added and the cells were incubated for 1 h (or overnight).

After incubation in primary antibody, the cells were washed and resuspended in 200 µl PBS / BSA / Saponin / $NaN_3$, followed by incubation with 2 µl of secondary antibody conjugated to a fluorochrome for 45-60 minutes in the dark (to prevent photobleaching) at RT. After incubation the cells were washed two times. The pellet was then re-suspended in 500 µl PBS/0.02% BSA/0.02% $NaN_3$, and the samples were analysed using a Becton Dickinson Fluorescent Activated Cell Sorter (FACS Calibur) with software supplied by Cell Quest.

## 2.7. Phospho-IκB Detection

### 2.7.1. SDS-PAGE

#### 2.7.1.1. Principle of SDS-PAGE

Electrophoresis is a process by which molecules are separated on the basis of their charge. The term itself describes the movement of a charged particle (proteins in the case of this project) under the influence of an electric field. These charged particles, which possess a positive or negative net charge, migrate towards the electrode of their opposite polarity (Wilson & Walker, 2005).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most used method to separate proteins according to their molecular weights (and therefore size). This type of electrophoresis utilises the anionic (negatively charged) detergent SDS to strongly bind to proteins and denature them, separating a protein into its basic subunits as well as unfolding each of those subunits, if any. This leaves us with negative charge rod-shaped proteins whose only difference between each other, asides from their polypeptide chain, is their size. Thus, these proteins (or subunits) will all move towards the positive electrode in electrophoresis (Wilson & Walker, 2005).

These proteins are then run through a cross-linked polyacrylamide gel under an electric current to separate them. The polyacrylamide gel acts as a molecular sieve, with the smaller protein moving faster, while the bigger proteins move slower (Wilson & Walker, 2005).

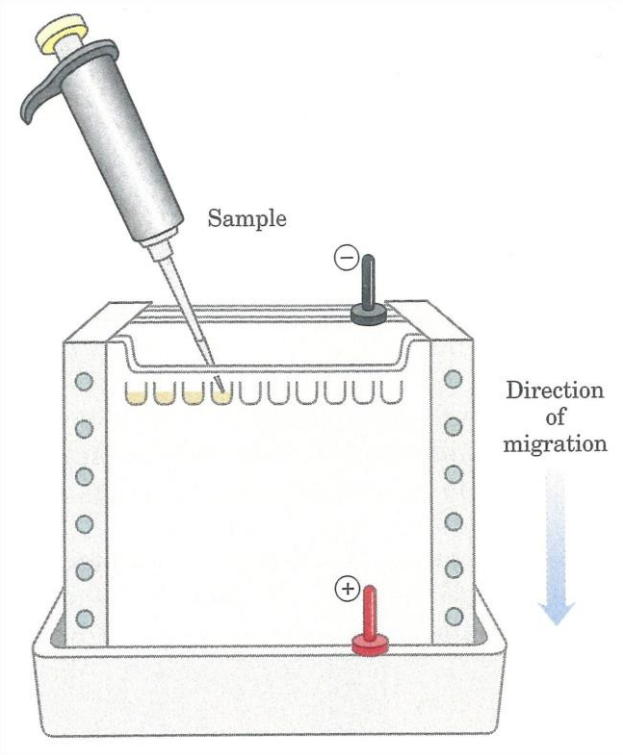


**Figure 2.5 –** An example of electrophoresis where different samples are loaded into the wells at the top of the polyacrylamide gel. The proteins (samples) move into the gel in the direction of migration (towards the positive end) when an electric current is applied **(Lehninger, et al., 1993)**.

After the proteins have been separated, the gel can be stained (with a dye such as Coomassie Blue) for identification of the bands or the proteins can be transferred to a porous membrane for another technique known as Western blotting (Wilson & Walker, 2005).

In this project, SDS-PAGE followed by western blotting was used to detect the presence of phospho-IκB in the cells' lysate. As explained in the introduction, IκB is an inhibitory protein that regulates NF-κB. TLRs activated by their ligand, interact with MyD88 (or TRIF), which induces the recruitment of downstream signalling molecules. This results in IκB being released from the NF-κB complex and getting phosphorylated, marking it for ubiquitination and degradation by proteosomes, and leads to the activation of NF-κB.

Once the apparatus was set up, 10% resolving gel was poured in between the glass plates up to the mark made previously. 10% ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) were only added once the gel was ready to be

poured, as they catalyse the polymerisation of the acrylamide gel. A thin strip of isobutanol (top layer) was added to the surface of the resolving gel, to remove any bubbles, and the gel was then left for approximately 45-60 minutes to polymerise. Once the resolving gel was set, the isobutanol was washed off with $dH_2O$, and 4% stacking gel was added all the way to the top of the cassette (again, 10% APS and TEMED were added once the gel was ready to be poured). A comb was inserted at this stage to create the wells, and the stacking gel was left to polymerise for 45-60 minutes.

Once set, the plates were unclipped from the casting stand and slid into a U-shaped gasket electrode assembly, short plate facing inwards, and placed into a tank. Approximately 1 inch of running buffer was added to the tank and filled between the plates.

2.7.1.2. Sample preparation for SDS-PAGE

To prepare the samples for running on the gel, 2X SDS-PAGE Reducing Sample Buffer was added to cells that had been incubated with different concentrations of V or F1 recombinant proteins or TLR ligands. The SDS in the reducing sample buffer is an anionic surfactant which both denatures secondary and non-disulphide-linked tertiary structures in proteins, and places a negative charge onto each protein in proportion to its mass. This ensures that each protein is linear and separated only by its MW. The β-mercaptoethanol present further denatures proteins by cleaving their disulphide bonds, thereby disrupting the tertiary and quaternary structure of them. The glycerol helps preserve the proteins at low temperatures, and weighs down the samples when loading them into the wells.

100 µl of each sample was added to separate Eppendorfs and boiled for 10 minutes, along with biotinylated SDS-PAGE standards (2 µl + 40 µl 2X SDS-PAGE Reducing Sample Buffer), to help denature the proteins further. The standards are a mixture of biotinylated proteins with consistent molecular weights, allowing for accurate molecular weight determination of immune detected proteins. 40 µl of the samples (10 µl of standards) were then loaded into the wells, and the apparatus was run

at a constant voltage of 200V for 45 minutes, or until the blue dye ran off the bottom of the gel.

### 2.7.2. Western blot

Western blotting is a technique used to transfer the proteins that have been separated by SDS-PAGE onto a nitrocellulose membrane, and then they are probed with antibodies. Using the electroblotting method, a sandwich of the gel (with the stacking gel trimmed off) and the nitrocellulose membrane was compressed in a cassette between two layers of blotting paper and pads pre-soaked in transfer buffer. The gel holder cassette was then placed into a tank transfer system together with an ice block, and the tank filled with transfer buffer. A constant current of 210 mA was applied for 60 minutes, electrophoretically transferring the proteins from the gel to the membrane.

#### 2.7.2.1. Blocking and primary antibody incubation

After the transfer was complete, the membrane was washed in 0.1% PBS-Tween. All washes and incubations were performed at RT on a rocking table, to ensure the membrane was fully covered, and the membrane was never left to dry. The membrane was then blocked for 60 minutes using 5% blocking reagent, and washed twice for 15 minutes with 0.1% PBS-Tween. The blocking reagent blocks any remaining hydrophobic binding sites, preventing the binding of the primary antibody to the membrane itself and thereby reducing background signal. The membrane was incubated for 60 minutes with a 1:1000 dilution of the primary antibody (Phospho-IκBα {Ser32} Rabbit mAb). After use, the primary antibody can be stored at -20$^{o}$ Celsius and reused.

2.7.2.2. Secondary antibody incubation

Two washes of 15 minutes with 0.1% PBS-Tween were then performed to remove excess (unbound) primary antibody, before the secondary antibody was added. A 1:2000 dilution of polyclonal Swine anti-Rabbit Ig horseradish peroxidase (HRP) was added to the samples, whilst a 1:1500 dilution of streptavidin-HRP conjugate was added to the standards, and left to incubate for 45-60 minutes. The membrane was then washed with 0.1% PBS-Tween for 2 hours, changing the solution every 15 minutes, to ensure all excess antibody had been removed before visualising the bands using enhanced chemiluminescence.

2.7.2.3. Enhanced chemiluminescence

The emission of light as a result of the dissipation of energy from a substance in an excited state, caused by a chemical reaction, is termed chemiluminescence. Enhanced chemiluminescence (ECL) involves the use of enhancers (such as phenols) to increase the light output and extend the light emission duration.

Detection of the bands was performed in a dark room, to avoid exposing the autoradiography film to light. Excess 0.1% PBS-Tween was drained from the membrane before placing it, protein side up, on a sheet of cling film in an X-ray cassette. An equal volume of ECL Reagent (GE Healthcare) was mixed together (1ml of each per membrane), added to the membrane, and incubated for 1 minute. Excess reagent was dabbed off, the membrane was turned over (protein side down), and sealed like an envelope in the cling film, smoothing out any air bubbles. The wrapped film was then placed protein side up. A sheet of autoradiography film was placed on top of the membrane, the cassette was closed and exposed for 2 minutes. The film was then developed immediately, and depending on the intensity and clarity of the bands seen, a new sheet of film could be exposed for a differing length of time.

2.7.2.4. Stripping and reprobing membranes

A membrane may be stripped of its bound primary and secondary antibodies, allowing it to be reprobed with different antibodies. To strip a membrane, stripping buffer was added (enough to cover the membrane), and incubated for 4 minutes at 37$^{o}$C in a shaker incubator. After washing at RT with 0.1% PBS-Tween 3 times for 10 minutes each, the membrane was blocked for 60 minutes using 5% blocking reagent, and washed twice for 15 minutes with 0.1% PBS-Tween. A different primary antibody can then be added, following the procedure above.

## 2.8. Cytokine analysis

To quantify the inflammatory response of the various cell types used in this study the levels of released cytokines were measured. Cytokines are the messengers that co-ordinate inflammation and give a direct representation of cellular response.

In this study the Human Th1/Th2 BD™ Cytometric Bead Array (CBA {BD Biosciences}) kit was used to measure cytokine concentrations. The Human Th1/Th2 CBA kit is capable of detecting six cytokines that play important roles in the human inflammatory response. These are Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumour Necrosis Factor-α (TNF-α) or Interferon-γ (IFN-γ) cytokines. This CBA analysis allows the fast and highly sensitive quantification of an array of cytokines in any one sample.

In order to bind cytokines in a sample, the beads are coated with capture antibodies specific for a particular cytokine. In the Human Th1/Th2 BD™ CBA kit there are six bead populations with different fluorescent emission wavelengths specific for IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ. Once the beads have bound to the cytokines a phycoerythrin (PE)-conjugated detection antibody mix is added, this is a mixture of PE-conjugated antibodies specific for each bead (Figure 2.6).

### 2.8.1. Cytometric Bead Array

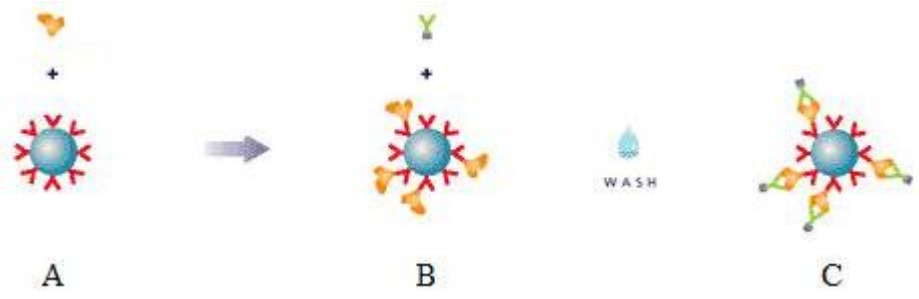


**Figure 2.6 –** BD™ Cytometric bead array (CBA) system. A) CBA beads (blue) bind cytokines (yellow) in sample. B) Phycoerythrin (PE)-conjugated detection antibody (green) is added. Solution is incubated for 3 hours and then the excess washed off. C) Cytokine can be seen sandwiched between bead and the PE detection antibody **(BD, 2012)**.

The Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur™) was used in conjunction with CellQuest software (Becton Dickinson) in order to run the samples. CBA Analysis Software (Becton Dickinson) was used to process the raw data to cytokine concentration (pg/ml) using a previously calibrated standard curve.

2.8.1.1. CBA protocol

To analyze the inflammatory response of any given sample the medium was tested using the Human Th1/Th2 BD™ Cytometric Bead Array (CBA {BD Biosciences}) kit.

For each sample 25 µl of bead mixture containing equal volumes of IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ beads was added to a Falcon flow tube. To this 25 µl of sample were added and the mixture vortexed briefly. 25 µl of phycoerythrin (PE)-conjugated detection antibody mixture was then added and vortexed briefly. The samples were then left out of light to incubate at room temperature for 3 hours, gently shaking every 30 minutes. 1 ml wash buffer (1X PBS) was then added to each sample which was then centrifuged at 1200 RPM for 5 minutes. The supernatant was poured off leaving the pellet undisturbed. 300 µl wash buffer (BD Biosciences) was added to each tube and then vortexed very briefly. The samples were assayed using the Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur™) used in conjunction with CellQuest software (Becton Dickinson). The cytokine concentration (pg/ml) was determined by data processing using CBA Analysis Software (Becton Dickinson).

## 2.9. Statistical Analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) software version 15.0 (SPSS, Inc.). Two sets of data were compared using a two-tailed, paired t-test. In this study a p value of less than 0.05 ($p < 0.05$) was considered significant. Throughout this thesis, *=p less than 0.05.

## 2.10. Confocal Microscopy

Confocal microscopy is a technique used to visualise the interaction and location of different proteins within cells. Conventional epifluorescence microscopy has the disadvantage that images from relatively thick cells are not very clear due to the image being composed of the fluorescence of the optical plane of interest as well as fluorescence from above and below the optical plane. (Wilson & Walker, 2005).

Confocal microscopy is a type of microscopy in which this problem is resolved by the microscope being capable of removing the out-of-focus fluorescence from the image, resulting in a sharper image to be viewed. This is referred to as optical sectioning (Wilson & Walker, 2005).

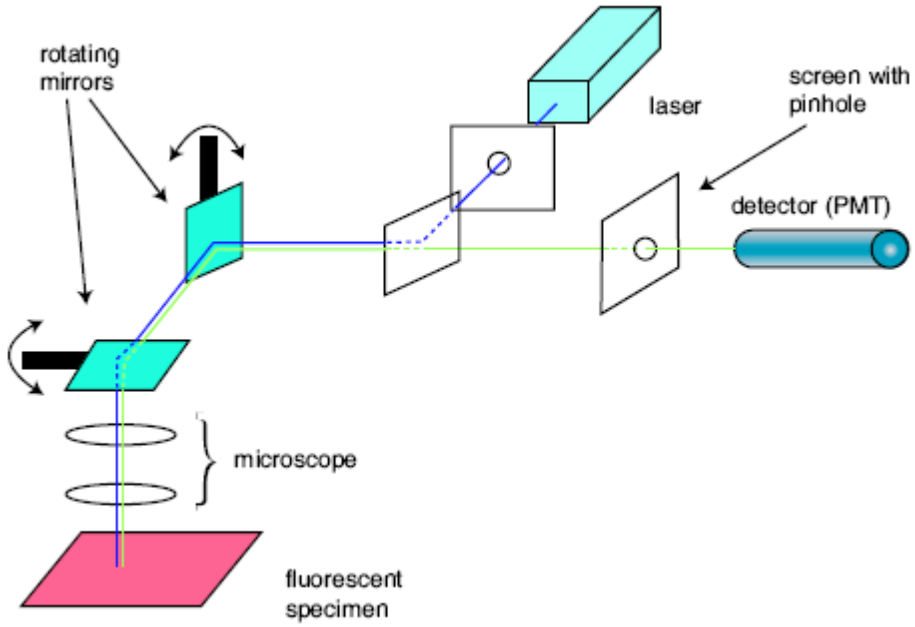


**Figure 2.7 –** Basic setup of a typical confocal microscope **(Semwogerere & Weeks, 2005)**.

The microscope used in this study was a Zeiss LSM 510 Confocal microscope using Zeiss LSM software.

2.10.1. Oregon Green-V or F1 conjugation

In order to be able to visualise V and F1 recombinant proteins within the cells, they were labelled with Oregon-Green. 1 mg of V or F1 recombinant protein was buffered exchanged into 1 M $NaHCO_3$, pH 8 using Pierce slide-a-lyser mini dialysis micro-tubes.

Following this, 5 mg of Oregon Green (OG) was dissolved in 500 µl of DMSO, creating a solution of OG (10 mg/ml). 500 µl of recombinant protein (1 mg/ml) was mixed with 250 µl of OG (10 mg/ml). The tube was sealed with parafilm, wrapped in aluminium foil and left to incubate away from light at 120 RPM and 37$^{o}$C for 3 hours.

In order to separate the conjugated protein from the unconjugated OG, a Sephadex PD-10 column (GE Healthcare) was used. Prior to separating the conjugated protein, the Sephadex column was equilibrated with 1X PBS. The conjugated protein was added to the Sephadex column and allowed to run through the column by the use of gravity alone. This results in the bigger conjugated OG-protein separating itself from the smaller unconjugated OG and eluting from the column first. Therefore, the first coloured fraction that appeared coming from the column was OG-protein, which was collected, wrapped in aluminium foil to protect it from the light and then stored in a refrigerator until needed.

2.10.2. Preparing poly-l-lysine slides

Slides were washed in alcoholic HCl for 1 hour and then washed 9 times with distilled water. Following this, the slides were placed in a 0.01% poly-l-lysine solution for 10 minutes, at room temperature and then dunked once in water. Finally, the slides were placed in an oven to dry at 70$^{o}$C for 25 minutes.

2.10.3. Labelling Mono Mac 6 cells on poly-l-lysine slides

Both direct and indirect immunofluorescence techniques were used to label Mono Mac 6 cells. Mono Mac 6 cells were either not stimulated or stimulated with V or F1 recombinant proteins and/or TLR ligands in 24-well plates as previously described. Following stimulation, the cells were collected and fixed by adding 300 µl of 4% PFA to each tube, resuspending the cells and leaving them to incubate for at least 15 minutes. The cells were then washed once with 500 µl of PBS.

A blocking solution of 100 µl of PBS/0.02% BSA/0.02% $NaN_3$/0.02% Saponin was then added to each tube and the cells were resuspended. 2 µl of the appropriate primary antibody was then added and the cells were left to incubate for a minimum of 1 hour.

Once incubation time with the primary antibodies was over, the tubes were centrifuged and washed once with 500 µl of 2X PBS. Following this, the cells were resuspended in 100 µl of PBS/0.02% BSA/0.02% $NaN_3$/0.02% Saponin and 2 µl of the appropriate secondary antibody was added and the tubes left to incubate for a minimum of 45 minutes but no more than 1 hour. The tubes were then washed twice with 500 µl of PBS/0.02% BSA/0.02% $NaN_3$/0.02% Saponin.

2.10.4. Preparing slides with samples

Once the cells were labelled, 20 µl of SlowFade was added to each of the samples and the cells well resuspended. Each sample was then transferred to a previously prepared poly-l-lysine slide (as described above) and a coverslip was carefully placed on each sample, taking care to make sure there were as few air bubbles as possible. The sample was then sealed by applying nail varnish around the edges of the coverslip and letting it dry. The slides were then ready to be viewed under the laser scanning confocal microscope.

# ***RESULTS***

# *Chapter 3 - V and F1 Antigen Expression and Purification*

## 3.1. V and F1 antigen purification

In order to investigate the effect that V and F1 antigen might have on the innate immune response, recombinant versions of V and F1 had to be produced. The plasmids containing the genes for GST-tagged LcrV and the *caf* operon (which contains the F1 gene) were transformed into appropriate strains of *E. coli* and kindly supplied by Professor R. Titball of the Ministry of Defence, Porton Down. The genes were then expressed using IPTG to drive promoter expression and the individual proteins were affinity purified as previously described (Sections 2.3 and 2.4).

The recombinant proteins were analysed to ensure protein purity and to determine levels of LPS contamination, the latter of which was removed prior to biological analyses.

## 3.2. Purification of V antigen

In order to produce recombinant V antigen, the recombinant LcrV plasmid (pVG110) with a glutathione S-transferase (GST) tag was used which was kindly provided by Prof. Richard Titball (Chemical and Biological Defense Establishment, Porton Down, Salisbury, UK). The plasmid was transformed into *E. coli* BL21 cells as previously described (Carr, et al., 2000).

The cloning and expression of the gene was done by Prof. Titball and colleagues as described in Carr et al 2000. Briefly, the *lcrV* gene (Genebank accession number AAA27641) was amplified by PCR from the chromosomal DNA of *Y. pestis* strain GB using 500 pmol of primers (Cruachem, Strathclyde, UK) homologous to the 5' and 3' ends of the gene (the 5' primer GATCGAATTC$^{+}$GAGCCTACGAACAAAACCCA and the 3' primer GGATCGTCGACTTA$^{*}$CATAATTACCTCGTGTCA), respectively and cloned into pGEX-6P-2 (GE Healthcare) to make the recombinant plasmid, pVG110.

*E. coli* BL21 cells containing the recombinant plasmid were cultured in L-broth containing 100 mg/ml ampicillin. Cultures were incubated with shaking (120 RPM at 37$^{o}$C until an $A_{600}$ of 0.3 was achieved; cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 5 h and harvested by centrifugation at 6500 RPM for 30 min

Harvested cells were resuspended in PBS and lysed by repeated freeze-thaw cycles. Crude cell extract was clarified by centrifugation at 2500 RPM for 30 min and the supernatant added to glutathione Sepharose columns, previously equilibrated with PBS. Unbound material was washed off with PBS. Cleavage buffer (2 ml) (50 mM Tris-HCl, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT) with PreScission™ Protease (30 µl) was added to the column and incubated at room temperature for 3 h. The column was washed with more cleavage buffer (3 ml) and the eluate was collected.

The eluate was concentrated and subsequently passed through a Profos Endotrap® blue 10 (Hycult Biotechnology) to ensure there was no LPS in the final solution. The HyCult LAL Chromogenic Endotoxin Quantitation Kit was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* amoebocyte lysate (LAL) assay, which was found to be none.

The preparations of purifed rV were analysed by SDS-PAGE followed by Coomassie blue staining. For Western blotting, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and detected using a V antigen specific monoclonal antibody (mouse anti-LcrV [Ab983] {Abcam}) at 1:1000 dilution followed by a goat anti-mouse IgG horseradish peroxidase conjugate at 1:2000 dilution.

The molecular weight of the protein was determined by SDS-PAGE and was found to be 38 kDa, which is within the predicted molecular weight range of native V antigen (Fig. 3.1). Although some lower molecular weight proteins were observed after Coomassie staining (which could be V antigen fragments), the major band observed was the 38 kDa V antigen.

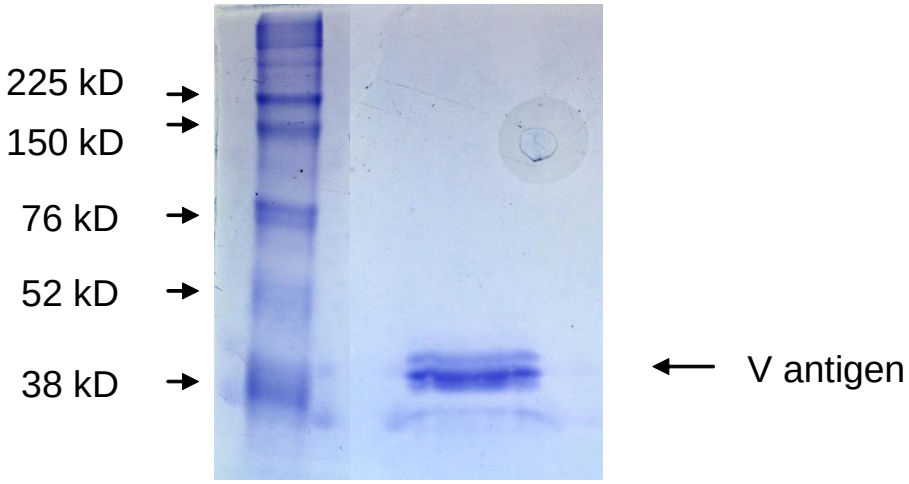


**Figure 3.1 – Coomassie blue stain for detection of purity of V antigen.** 5 μg of V antigen were analysed by SDS-PAGE and subsequently stained with Coomassie blue stain. Molecular weight markers were used to determine the correct position of the molecular weight of the bands indicating presence of the desired protein.

A strong band appeared around 38 kDa, which suggests that there were no other proteins present except V antigen. In order to verify the identity of the 38 kDa band observed with the Coomassie blue stain, a western blot was performed using an anti-V antigen specific antibody (Figure 3.2). It was shown that the band identified in the Coomassie blue stained gel, was indeed V antigen. Some minor bands of lower and higher molecular weight that appeared on the western blot must represent fragments and aggregates of V antigen since they are recognized by the mAb specific for V antigen.

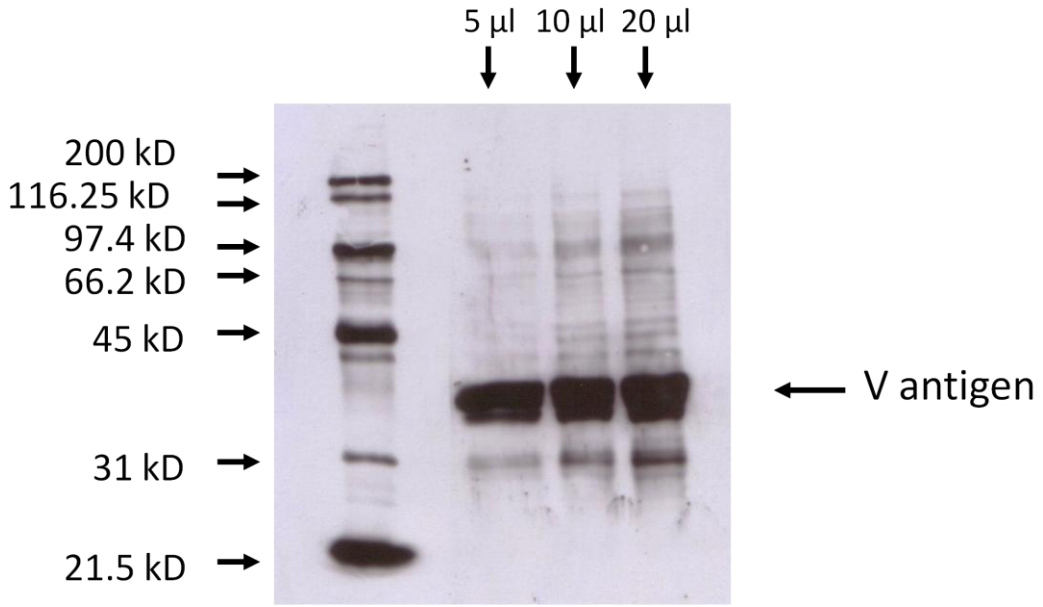


**Figure 3.2 – Western Blots for detection of V antigen at different concentrations.** Molecular weight markers were used to determine the correct position of the molecular weight of the bands indicating presence of the desired protein. The loading volumes in the wells were 5 µl, 10 µl and 20 µl, respectively, of the purified solution.

### 3.3. Purification of F1 antigen

Similarly with the V antigen, in order to produce recombinant F1 antigen, The F1 plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6 was used which was kindly provided by Prof. Richard Titball (Chemical and Biological Defense Establishment, Porton Down, Salisbury, UK). The plasmid was transformed into *E. coli* JM101 cells as previously described (Miller, et al., 1998).

The cloning and expression of the gene was done by Prof. Titball and colleagues as described in Titball, et al (1997). Briefly, two DNA fragments, which together included the *Y. pestis caf* operon, were amplified by PCR with *Y. pestis* MP6 template DNA and oligonucleotide primer pair FIOU2 (5'TGCCCCGGGAATTCCGAACATAA ATCGGTTCAGTGGCC3') and M4D (5'GGCGTATTCCTCGCTAGCAATGTTTAA CG3') or M3U (5'ATCGTTAAACATTGCTAGCGAGGAATACGCC3') and FIOD2 (5'GATA GATCTGTCGACTGAACCTATTATATTGCTTCGCGC3'). The amplified DNA fragments were ligated to form a whole *caf* operon which was cloned into low-copy-number plasmid pLG339 to make the recombinant plasmid, pAH34L.

In contrast to the LcrV, the F1 encoding gene did not have an encoded tag attached to it, thus, in order to purify it, affinity chromatography was used. E. coli JM101 containing plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6, was cultured in L-broth containing 10 mg/ml kanamycin. Cultures were incubated with shaking (120 RPM) at 37$^{o}$C until an $A_{600}$ of 0.3 was achieved; cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were incubated for a further 5 h and harvested by centrifugation at 6500 RPM for 30 min.

Harvested cells were resuspended in PBS and lysed by repeated freeze-thaw cycles. Crude cell extract was clarified by centrifugation at 2500 RPM for 30 min at 4$^{o}$C and the supernatant was added to HiTrap columns conjugated with F1-specific mAb (mouse anti-F1 antigen [YPF19] {Abcam}). The unbound proteins were washed off with PBS and bound protein was eluted using 0.1 % trifluoacetic acid pH 5. Eluted F1 protein were collected, buffer-exchanged and concentrated prior to endotoxin removal using Profos Endotrap® blue 10 (Hycult Biotechnology) in order to ensure there was no LPS in the final solution. The HyCult LAL Chromogenic Endotoxin Quantitation Kit

was used in order to measure the amount of endotoxin in the purified protein sample using the *Limulus* amoebocyte lysate (LAL) assay, which was found to be none.

In order to verify the level of purity of F1 antigen the protein was analysed by SDS-PAGE and subsequently Coomassie blue stain (Figure 3.3). It was shown that only one strong band appeared around 24 kDa, which suggests that there are no other proteins in the gel except F1 antigen. Some higher molecular bands also appeared which could be F1 multimers.

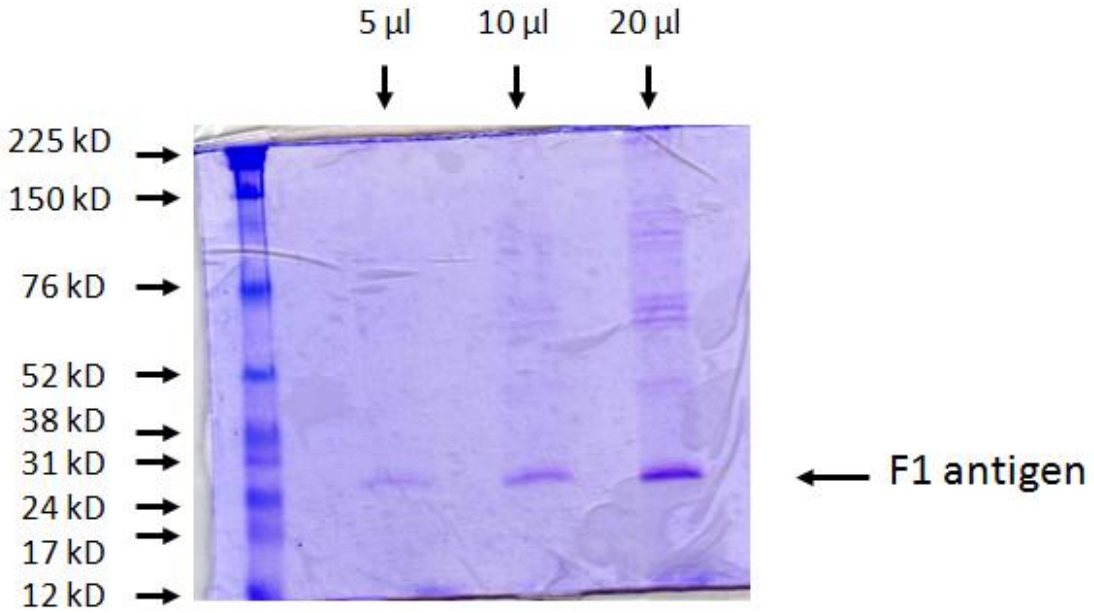


**Figure 3.3 – Coomassie blue stain for detection of purity of F1 antigen.** 5 µl, 10 µl and 20 µl of F1 antigen were analysed by SDS-PAGE and subsequently stained with Coomassie blue stain. Molecular weight markers were used to determine the correct position of the molecular weight of the bands indicating presence of the desired protein.

In order to verify the identity of the 24 kDa band observed with the Coomassie blue stain, a western blot was performed using an anti-F1 antigen specific antibody (Figure 3.4). It was shown that the band identified in the Coomassie blue stained gel, was indeed F1 antigen. The higher molecular weight proteins observed using Coomassie blue stain were not detected by Western blotting, indicating that they do not contain the epitope recognised by mouse anti-F1 antigen [YPF19] antibody. They are probably F1 aggregates where the epitope recognised by the mAb is masked.

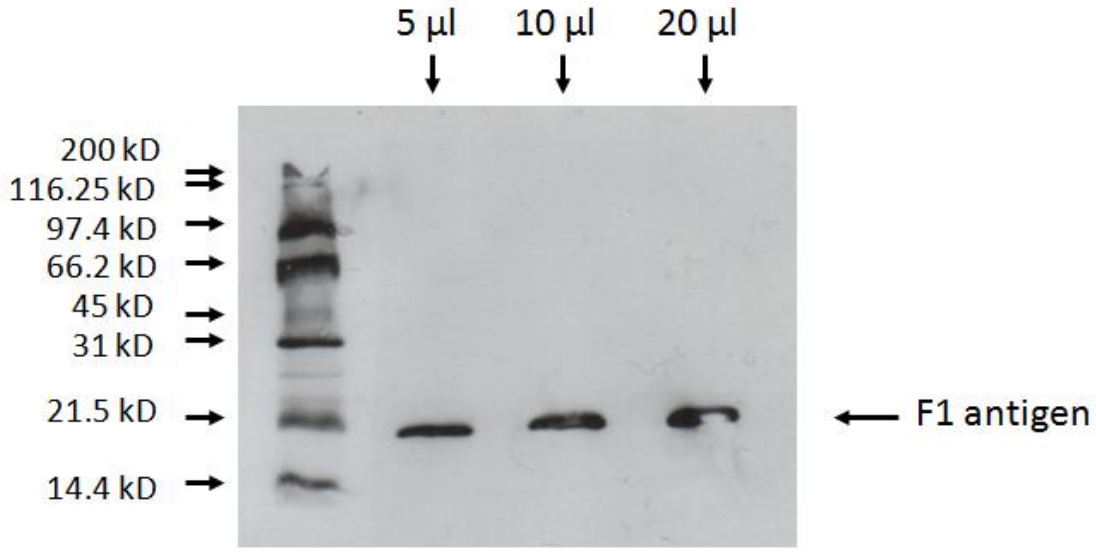


**Figure 3.4 – Western Blots for detection of F1 antigen at different concentrations.** Molecular weight markers were used to determine the correct position of the molecular weight of the bands indicating presence of the desired protein. The loading volumes in the wells were 5 µl, 10 µl and 20 µl, respectively, of the purified solution.

## 3.4. Conclusions

In order to investigate the effects of V and F1 antigens from *Y. pestis* on human monocytes, recombinant proteins had to be produced. Prof. Richard Titball kindly provided us with the recombinant LcrV plasmid (pVG110) with a glutathione S-transferase (GST) tag. The plasmid was transformed into *E. coli* BL21 cells as previously described (Carr, et al., 2000). The F1 plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6 was also provided by Prof. Richard Titball. The plasmid was transformed into *E. coli* JM101 cells as previously described (Miller, et al., 1998).

The LcrV protein was encoded with a glutathione S-transferase (GST) tag, thus it was straightforward to isolate the protein from the crude *E. coli* cell extract. LcrV recombinant proteins were isolated using glutathione sepharose columns which have an affinity for the GST tag, and subsequently eluted using PreScission™ Proteases. The eluted protein was buffer-exchanged, concentrated and all traces of endotoxin removed by passing the protein through a Profos Endotrap® blue 10 (Hycult Biotechnology) column. In order to verify that all the endotoxin was removed, the *Limulus* amoebocyte lysate (LAL) assay was used, which found no traces of endotoxin.

The preparations of purified recombinant V antigen (rV) were analysed by SDS-PAGE followed by Coomassie blue staining. It was shown that the molecular weight of the protein was 38 kDa, which is the predicted molecular weight of native V antigen. Some lower molecular weight proteins were observed after Coomassie staining (which could be V antigen fragments), the major band observed was the 38 kDa V antigen. Western blotting also verified that the main protein band at 38 kDa was V antigen. Smaller fragments, as well as aggregates, also seemed to be present.

In contrast to the LcrV, the F1 encoding gene did not express a His, GST or other tag and thus in order to purify it affinity chromatography was used. *E. coli* JM101 containing plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6, was cultured in L-broth containing 10 mg/ml kanamycin.

The recombinant F1 protein was isolated from the crude *E. coli* cell extract using HiTrap columns conjugated with F1-specific mAb (mouse anti-F1 antigen [YPF19]

{Abcam}). The unbound proteins were washed off with PBS; bound protein was eluted using 0.1 % trifluoacetic acid pH 5. Eluted F1 proteins were collected, buffer-exchanged and concentrated prior to endotoxin removal using Profos Endotrap® blue 10 (Hycult Biotechnology) in order to ensure there was no LPS in the final solution. In order to verify that all the endotoxin was removed, the *Limulus* Amebocyte Lysate (LAL) assay was used, which found no traces of endotoxin. This is particularly important as the recombinant needs to be endotoxin-free in order to be able to assess its effects on human monocytes.

The preparations of purifed recombinant F1 antigen were analysed by SDS-PAGE followed by Coomassie blue staining. It was shown that the molecular weight of the protein is 21 kDa, which is close to the predicted molecular weight of native F1 antigen of 17.5 kDa. Some higher molecular weight proteins were observed after Coomassie staining, which could be F1 antigen aggregates, as F1 is known to exist in higher oligomers (Miller, et al., 1998; Tito, et al., 2001; Zavialov, et al., 2003). Western blotting also verified that the main protein band at 21 kDa was F1 antigen, whereas the higher fragments were not recognised by the F1-specific mAb (mouse anti-F1 antigen [YPF19] {Abcam}), possibly because the epitope that the antibody is recognising is masked in higher oligomers.

The initial stage of this study was to generate recombinant V and F1 proteins and characterise them. In order to investigate the effects of V and F1 antigens on the innate immune responses, recombinant proteins that were pure and endotoxin free had to be produced. Once this was achieved the effects of these proteins on the innate immune response were investigated.

# *Chapter 4 - Expression Levels of Toll Like Receptors In Human Monocytes In Response to V antigen*

## 4.1. *Y. pestis* V antigen as an innate immune modulator

*Y. pestis*, the causative agent of the three main plague pandemics has been responsible for over 200 million deaths throughout known history. The bacterium has been so successful because it encodes several virulence factors, one of which is LcrV. *Yersinia pestis* V antigen (LcrV) is a multifunctional protein and has been shown to exhibit immunomodulatory features, such as inducing secretion of the anti-inflammatory cytokine IL-10 and inhibiting neutrophil chemotaxis, and shown to be vital in translocating other virulence effectors into eukaryotic cells. It also possesses immunogenic properties, with anti-V immunoglobulins being detected in the serum of *Y. pestis*-infected patients. It is the proposal of this study that this multifunctional protein modulates the innate immune system in additional ways not yet discovered. One of the first things that were verified was whether recombinant LcrV derived from *Y. pestis* affects pattern recognition receptor expression in human monocytes, in particular Toll-like receptor expression.

## 4.2. Effect of LcrV on TLR expression

In order to determine the effect that *Y. pestis* V antigen has on the expression levels of several TLRs, Mono Mac 6 cells were stimulated with three different concentrations of V antigen (5 µg, 50 µg and 100 µg) over a 24-hour period (1h, 4h, 8h and 24h). The expression levels of the receptors at certain time points (1h, 4h, 8h and 24h) were detected using indirect immunofluorescence and flow cytometry. In addition to TLR expression, the expression levels of CD36 were also determined as it is a co-receptor of TLR2. The expression levels of these receptors of unstimulated Mono Mac 6 cells were used as a baseline control of Mono Mac 6 receptor expression levels prior to stimulation.

Initially, Mono Mac 6 cells were stimulated with 5 µg of V antigen (Figure 4.1). It was shown that all the TLRs were expressed on Mono Mac 6 cells with the exception of TLR1 which was found to emit a low fluorescent intensity, meaning that it is expressed in low amounts in these cells. V antigen seemed to induce downregulation of all TLRs within the first hour of stimulation, whereas by 4 hours the level of expression seemed to have recovered to levels higher than the basal ones, with the exception of TLR5 and TLR9, whose levels return to normal. A downregulation reoccurred by the 8 hours timepoint down to below the basal state but by the 24 hours timepoint the expression level of most TLRs had returned to basal levels.

In contrast to TLRs, CD36 was shown to be upregulated in response to V antigen. This upregulation occurred 4 hours after stimulation with V antigen and by 8 hours the expression level dropped significantly.

Therefore it seems that V antigen modulates PRR receptor expression and in this way manipulates the innate immune response. In the case of TLRs, it seems to downregulate, suggesting that it "dampens" or inhibits the response. In contrast, in the case of CD36 is seems to augments its expression early on in the infection, possibly promoting the internalization of *Y. pestis*, since CD36 is a scavenger receptor.

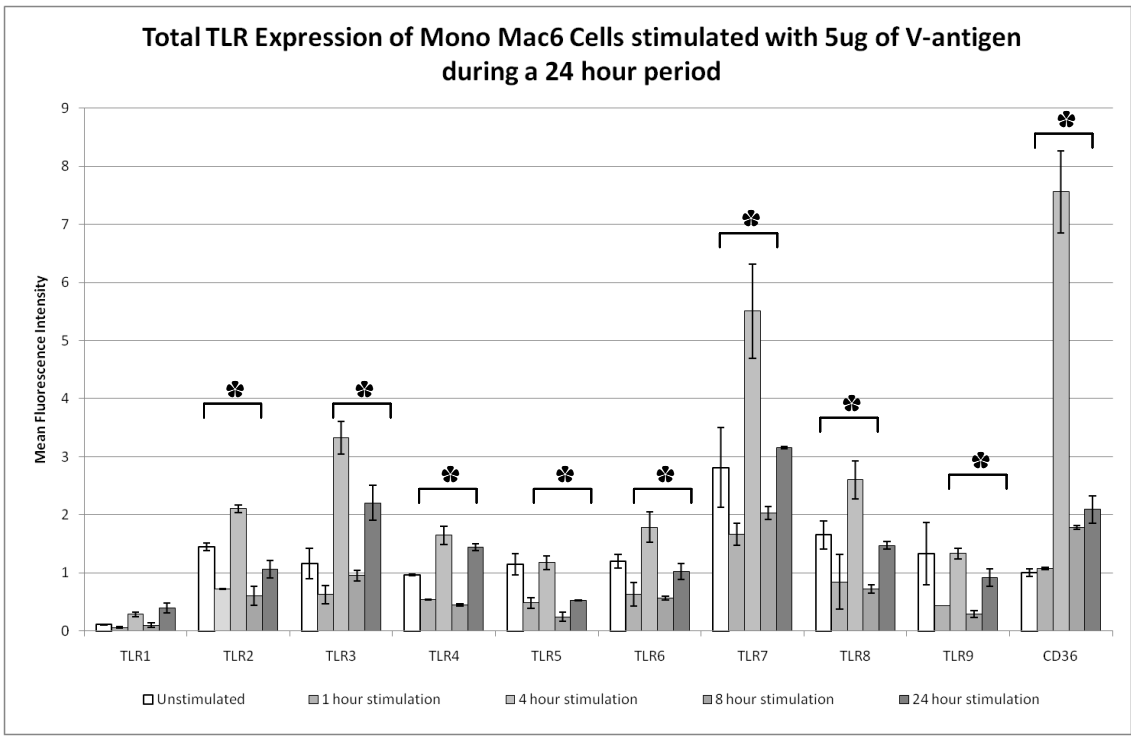


**Figure 4.1 – PRR expression levels in response to 5 µg of V antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 on Mono Mac 6 cells stimulated with 5 µg of V antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of Mono Mac 6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) difference in expression in comparison to unstimulated cells.

## 4.3. Effect of different concentrations of LcrV on TLR expression

In order to test whether the effect of V antigen on TLR expression is concentration-dependent, the effect of higher concentrations of V antigen using this protein at 50 µg (Figure 4.2) and 100 µg (Figure 4.3) on Mono Mac 6 cells was investigated.

It was shown that these higher concentrations of V antigen had the same overall effect on TLR and CD36 expression, except for TLR3, which showed distinct upregulation. The same trend of initial downregulation of the other TLRs was observed, whereas in the case of CD36 and TLR3 there was only upregulation (Figure 4.2 and 4.3). It is possible that since TLR3 signals differently compared to the other TLRs that somehow the protein affects it in a different way. V antigen could possibly be targeting the MyD88-dependent signalling pathway and since TLR3 is signalling via the MyD88-independent pathway it is not targeted. The only other noticeable difference when the cells were stimulated with higher concentrations of V antigen was that the overall expression level of all PRRs was reduced. For example when 5 µg of V antigen were used, the maximum fluorescence intensity for TLR7 was 5.505 ± 0.813 at 4 hours, whereas when stimulated with 50 µg and 100 µg it was 2.235 ± 0.021 and 2.115 ± 0.205, respectively (less than half the overall fluorescent intensity).

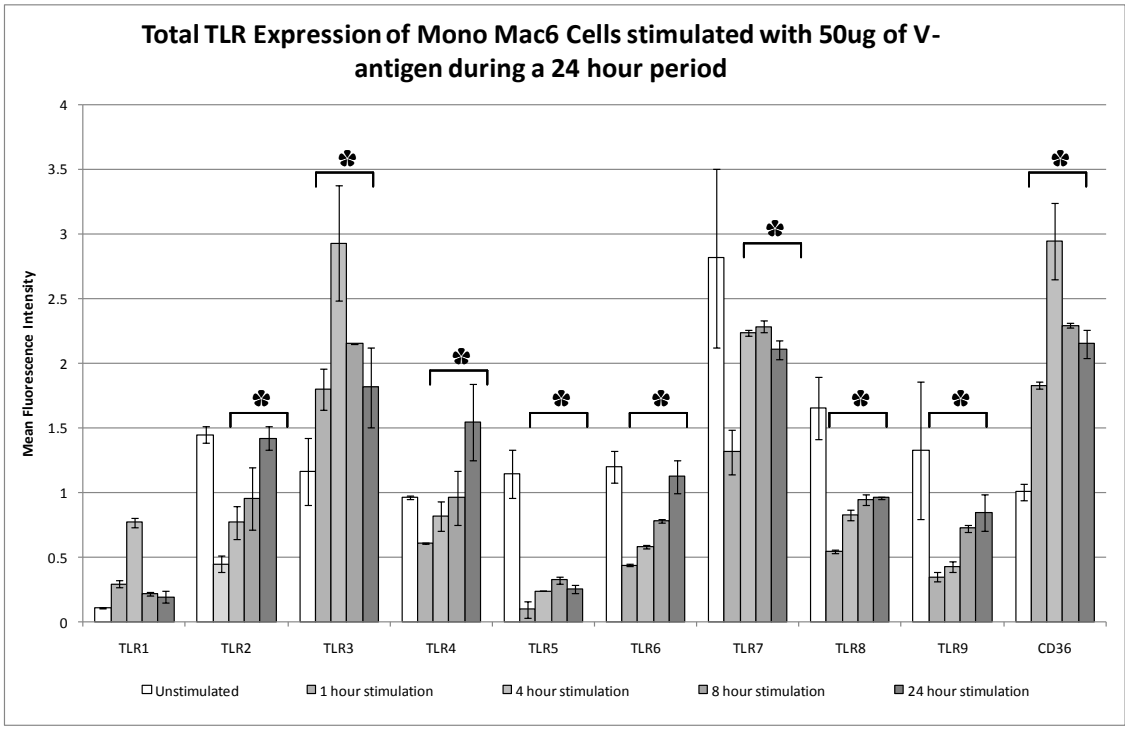


**Figure 4.2 – PRR expression levels in response to 50 µg of V antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 of Mono Mac 6 cells stimulated with 50 µg of V antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of MonoMac6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) difference in expression in comparison to unstimulated cells.

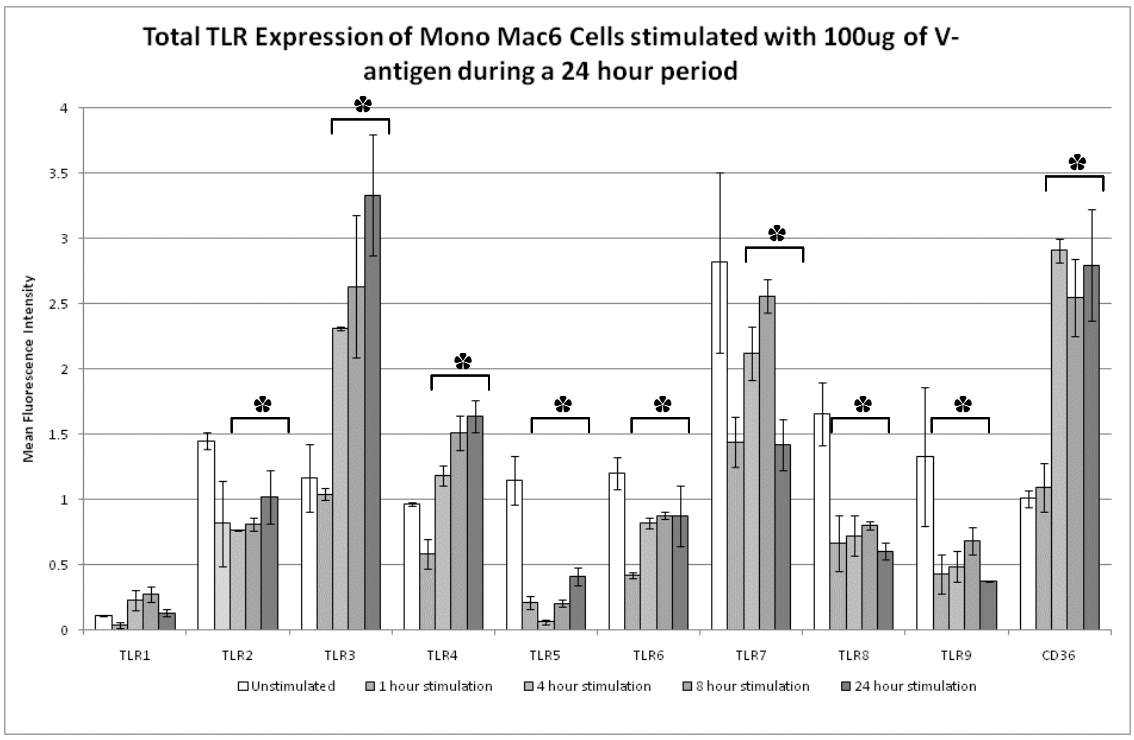


**Figure 4.3 – PRR expression levels in response to 100 µg of V antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 of Mono Mac 6 cells stimulated with 100 µg of V antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of Mono Mac 6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) difference in expression in comparison to unstimulated cells.

## 4.4. Conclusions

*Yersinia pestis* V antigen (LcrV) is a multifunctional protein and has been shown to exhibit immunomodulatory features, such as inducing secretion of the anti-inflammatory cytokine IL-10 and inhibiting neutrophil chemotaxis, and shown to be vital in translocating other virulence effectors into eukaryotic cells. One of the aims of this study was to investigate the effects of V antigen on innate immune responses.

Mono Mac 6 cells were stimulated with different concentrations of recombinant V antigen and the expression levels of TLRs over a 24-hour period were investigated. The results show that V antigen was able to modulate PRR expression. V antigen initially induced a downregulation of most TLRs within 1 hour of stimulation, thus suggesting that *Y. pestis* uses V antigen in order to "dampen" the innate immune response. This could be an evasion strategy that *Y. pestis* employs in order to dampen the innate immune response with the secretion of V antigen and then infect the host cells without any resistance.

In addition, it could be that V antigen can induce TLR clustering and thus promote rapid internalization of the receptors, therefore the observed downregulation in the level of expression observed within 1 hour. Since there was no time to perform PRR mRNA studies, it cannot be put aside the possibility that, instead of levels of PRR expression or solely levels of PRR expression, the difference in fluorescence intensity observed between samples is due to the epitope which the primary antibodies recognize for their specific receptors being hidden and therefore blocking the antibodies from binding and recognizing the presence of these receptors. Therefore, these results might actually be showing the presence of TLRs (and CD36) which have the primary antibodies' epitopes free and not those who happen to have these epitopes hidden or even a combination of that as well as receptor expression levels. Possibilities that might hide these epitopes include TLRs clustering with receptors (TLRs and others) as well as binding to other molecules involved in receptor activation or even V antigen binding to the receptor and hiding the epitope site in the process.

The TLR expression levels of the cells stimulated with 5 μg of V antigen show an initial downregulation at the 1 hour time point, followed by a pattern of upregulation

followed by downregulation throughout the rest of the time points collected. This up-and-down pattern might be possibly occurring as a consequence of the cells attempting to restore TLR expression levels to normal ('basal') levels but constantly coming into contact with V antigen that dampens the response and thus forces the receptor downregulation observed.

In the flow cytometry results from cells stimulated with 50 µg of V antigen, the results observed show the initial downregulation for most TLRs previously seen with the slightly lower concentration of 5 µg of V antigen followed by a steady increase throughout the rest of the time points collected. This could be a sign that the slightly higher concentration is having a stronger dampening effect on the expression of the affected TLRs and, instead of the overcompensating pattern observed with the lower concentration, we have a much more subdued and prolonged attempt by the cells to restore expression levels to basal state levels.

The only TLR that was found not to be downregulated by *Y. pestis* V antigen was TLR3. Its expression tended to undergo upregulation, rather than downregulation, with 50 µg and 100 µg of V antigen stimulation. It is possible that V antigen interferes only with the MyD88-dependent pathway in an inhibitory fashion but not the TRIF-dependent pathway, thus the downregulation of all TLRs, except TLR3 which does not utilise the MyD88-dependent pathway. Since it was observed that there is TLR3 upregulation, it is possible that V antigen dampens the MyD88-dependent pathway causing TLR downregulation and thus the TRIF pathway remains unaffected and tries to compensate by upregulating TLR3 and thus MyD88-independent signalling.

In contrast to TLRs, it was shown that V antigen upregulated CD36 expression in the early stages of incubation with Mono Mac 6 cells, possibly facilitating in this way the rapid internalization of *Y. pestis* within the cells. It is also interesting to note that the pattern of CD36 expression levels observed is very similar to that of TLR3, suggesting, perhaps, a link between the two receptors or, at least, in the way they are affected by V antigen stimulation.

It is interesting to note that four hours after stimulation with exogenous V antigen, PRR receptor level seemed to recover and reach levels even above the basal ones

observed. It is possible that V antigen is produced early on in the infection in order to "dampen" the innate immune system and hijack its machinery for internalization and four hours post stimulation, with no more addition of exogenous V antigen, the cell attempts to return to a basal state.

# *Chapter 5 - NF-κB Activation and Cytokine Secretions In Human Monocytic Cells In Response to V antigen*

## 5.1. Activation of the NF-kB signalling cascade

As previously mentioned, one of the consequences of activation of TLRs is the expression of genes via NF-κB which, normally, is sequestered by unphosphorylated IκB. One of the most common signalling pathways from TLR activation leads to the phosphorylation of IκB and therefore release of NF-κB. To verify whether this occurs and, therefore, whether cytokine secretion in this instance was linked to NF-κB activation, Mono Mac 6 cells were stimulated in serum-free medium with different concentrations (5 µg, 50 µg and 100 µg) of *Y. pestis* V antigen and the supernatant was collected at certain timepoints within a 24-hour period (1h, 4h, 8h and 24h). The supernatants from these samples were frozen and stored for later cytokine assays while the cells were lysed and the contents separated by size via SDS-PAGE. These proteins were then transferred to a membrane and western blotting was performed in order to detect the phosphorylated form of IκB.

Furthermore, in order to determine whether V antigen influenced the cytokine response to known TLR ligands, Mono Mac 6 cells were also stimulated in serum-free medium with certain PAMPs (LPS, LTA, Poly:I-C, ssRNA and CpG DNA) for 1 hour as well as pre- and post-treated with 5 µg of V antigen. These were also collected, the supernatant frozen and stored for cytokine assays while the cells were lysed and used to verify the presence of phospho-IκB.

## 5.2. Presence of phosphorylated IκB in response to V antigen

In order to determine whether *Y. pestis* V antigen promotes NF-κB activation, cells were exposed to V antigen and lysed at different time points (1h, 4h, 8h and 24h). The protein complex NF-κB is the transcription factor that is involved in the cellular response to stress and, of particular interest to this work, the presence of cytokines. The presence of phospho-IκB in the cell lysate indicates the transcription of proinflammatory cytokines. Western blotting was utilised in order to determine the presence of the phosphorylated form of IκB.

Initially, whether V antigen itself can trigger NF-κB activation was investigated, thus cell lysates that were only stimulated with V antigen at different time points (Figure 5.1) were analysed. It was shown that V antigen could trigger some NF-κB activation on its own.

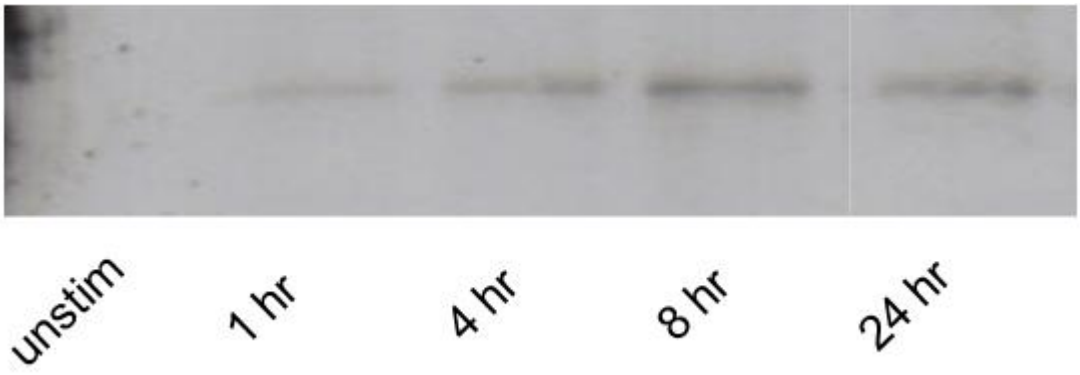


**Figure 5.1 – Presence of phosphorylated IκB in cells stimulated with V antigen at different time points.** Mono Mac 6 cells were stimulated with 5 µg of V antigen and lysed at 0, 1, 4, 8 and 24 hours. Cell lysates were analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 5.3. Presence of phosphorylated IκB in response to PAMPs

Since V antigen has already been shown to be able to modulate TLR expression and has been shown in other studies to modulate innate immune responses (Leary, et al., 1995; Welkos, et al., 1998; Reithmeier-Rost, et al., 2004), experiments were performed to expand these observations. In order to test this hypothesis, whether V antigen is able to modulate NF-kB activation in response to TLR ligands was investigated.

Initially NF-κB activation in response to PAMPs was investigated. It was shown that there was a strong band corresponding to the phosphorylated form of IkB in response to LPS and LTA and to a lesser extent in response to ssRNA, CpG DNA and Poly:I:C (Figure 5.2).

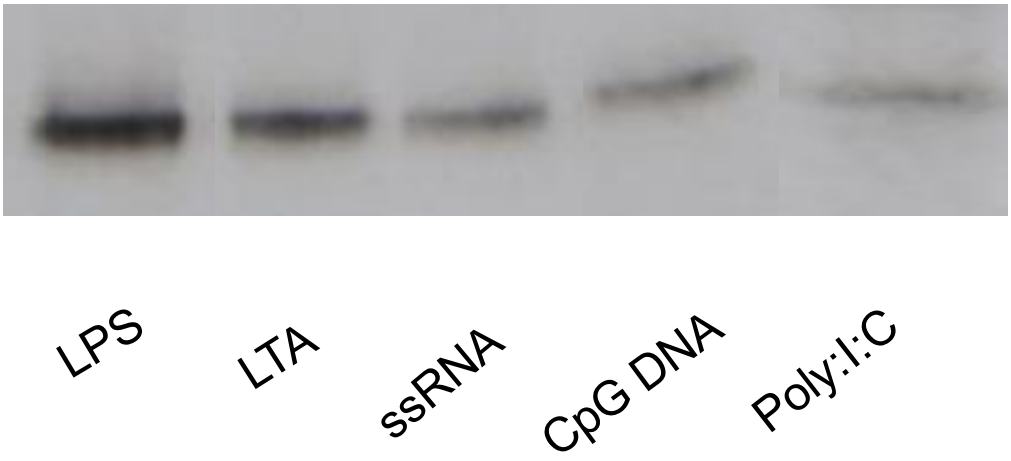


**Figure 5.2 – Presence of phosphorylated IκB in cells stimulated with PAMPs.** Mono Mac 6 cells were stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for 1 hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 5.4. Presence of phosphorylated IκB in response to PAMPs pre- and post-treated with V antigen

Once it was established that the different PAMPs could trigger the NF-κB signalling cascade, whether V antigen was able to modulate this response was investigated.

Initially, the effect that pre-treatment with V antigen will have on NF-κB activation was investigated. It was shown that the addition of V antigen inhibited the activation in response to the PAMPs. There was still some activation in response to LPS and LTA, but V antigen was able to completely inhibit activation in response to ssRNA, CpG DNA and Poly:I:C (Figure 5.3). Similar results were obtained when the cells were first stimulated with the PAMPs and subsequently V antigen was added (post-stimulation) (Figure 5.4).

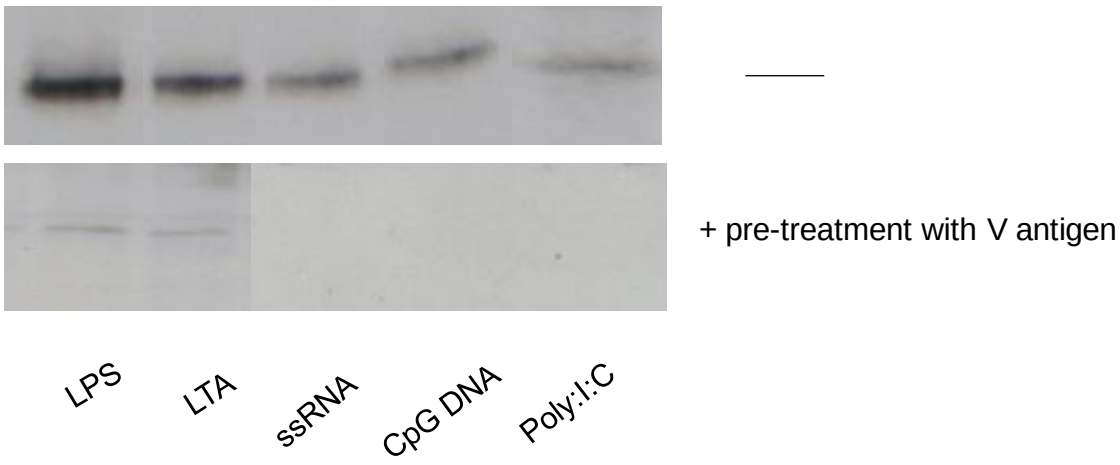


**Figure 5.3 – Presence of phosphorylated IκB in cells pre-treated with V antigen and subsequently incubated with PAMPs.** Mono Mac 6 cells were pre-treated with 5 µg of V antigen for 1 hour and subsequently stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for a further hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

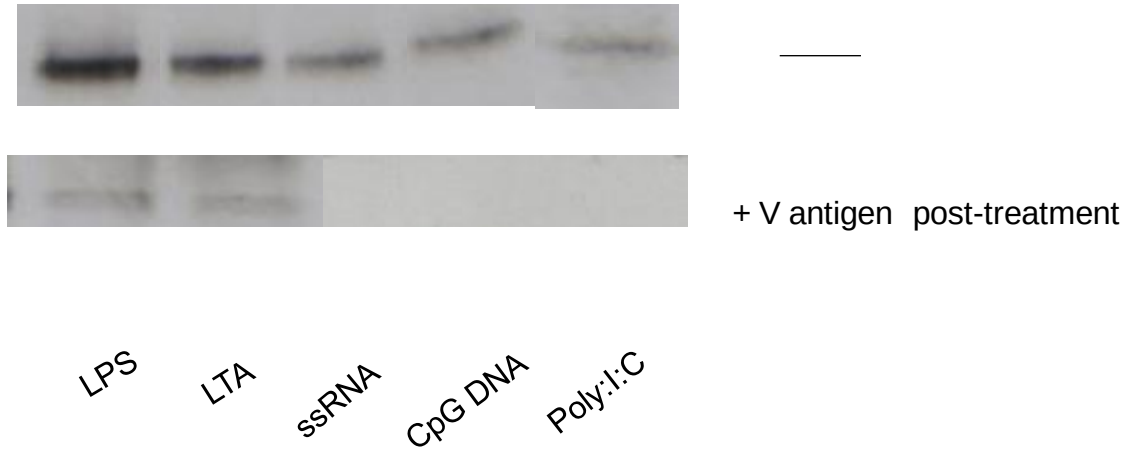


**Figure 5.4 – Presence of phosphorylated IκB in cells post-treated with V antigen.** Mono Mac 6 cells were stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for 1 hour and subsequently post-treated with 5 µg of V antigen for a further hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 5.5. Cytokines secreted by cells during a 24-hour period

The supernatants collected at different times from the Mono Mac 6 cells stimulated with different concentrations (5 µg, 50 µg and 100 µg) of *Y. pestis* V antigen were assayed for cytokine concentration. The presence of cytokines was measured using a Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit, which is capable of detecting TNF-α, IFN-γ, IL-2, IL-4, IL-6 and IL-10.

Initially TNF-α secretion in response to different concentrations of V antigen (5, 50 and 100 µg) as well as different PAMPs was studied. It was shown that V antigen on its own did not stimulate TNF-α secretion at any concentration tested. In contrast, the PAMPs and in particular LPS and LTA stimulated significant production of TNF-α over a 24 hour period, with a peak at 4 hours following stimulation (Figure 5.5).

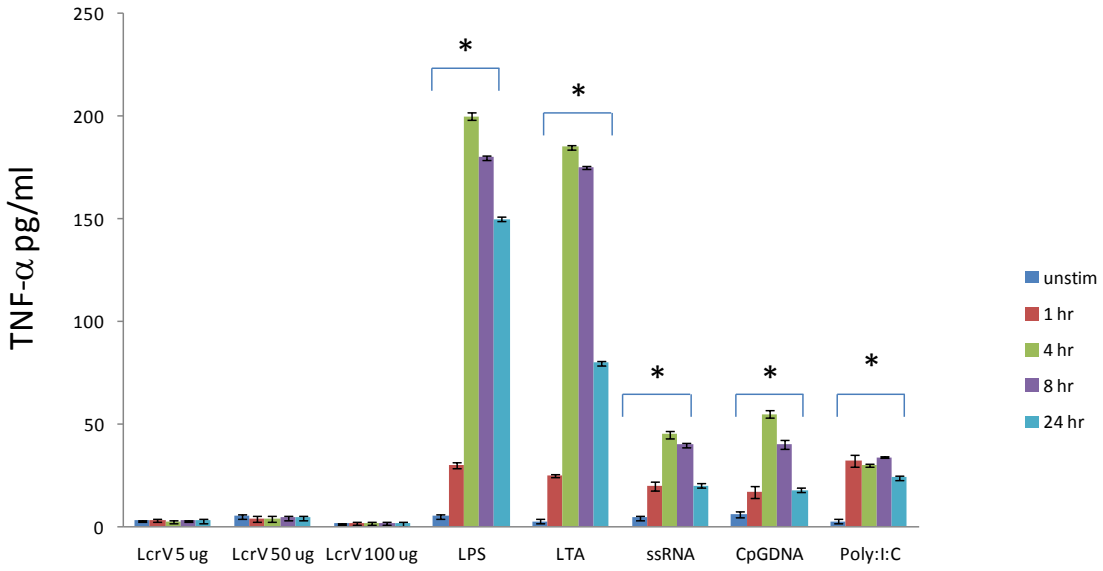


**Figure 5.5 – Secretion of TNF-α in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3). Asterisks indicates statistically significant ($p < 0.05$) secretion of cytokine in comparison to unstimulated cells.

In addition, IL-2 (Figure 5.6), IL-4 (Figure 5.7), IL-6 (Figure 5.8) and IFN-γ (Figure 5.9) secretion was also tested. All four cytokines were found not to be secreted

in response to the different ligands. While this was expected for IL-2, IL-4 and IFN-γ as monocytes do not secrete these cytokines, they are, however, known to secrete IL-6.

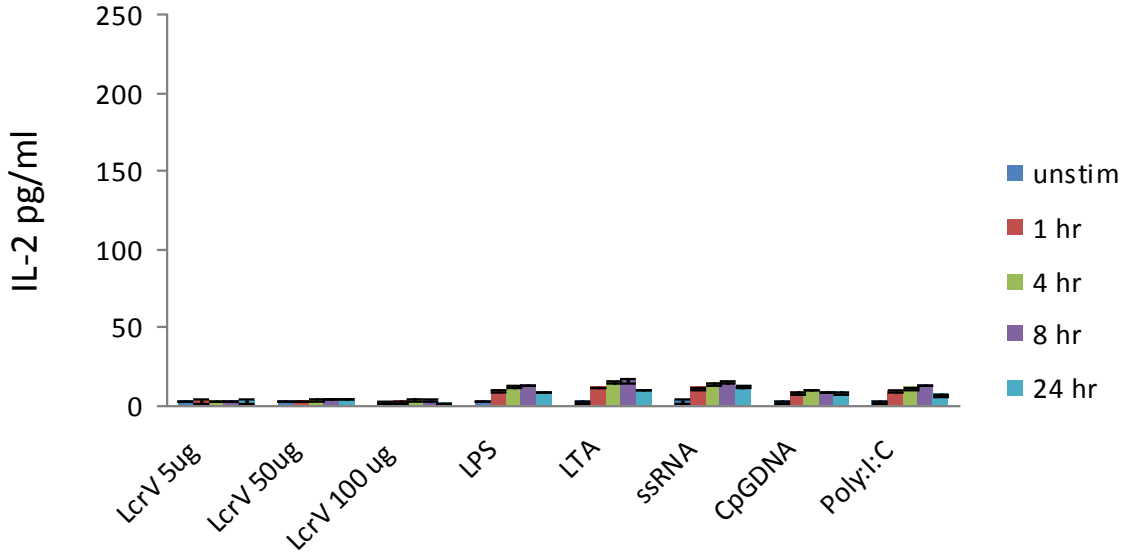


**Figure 5.6 – Secretion of IL-2 in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

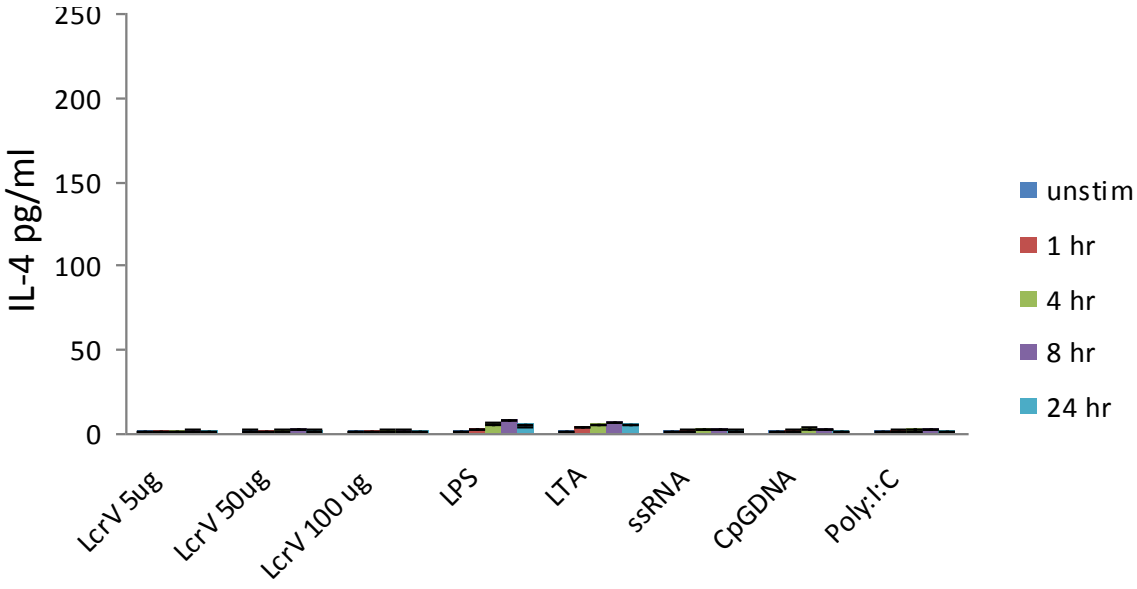


**Figure 5.7 – Secretion of IL-4 in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

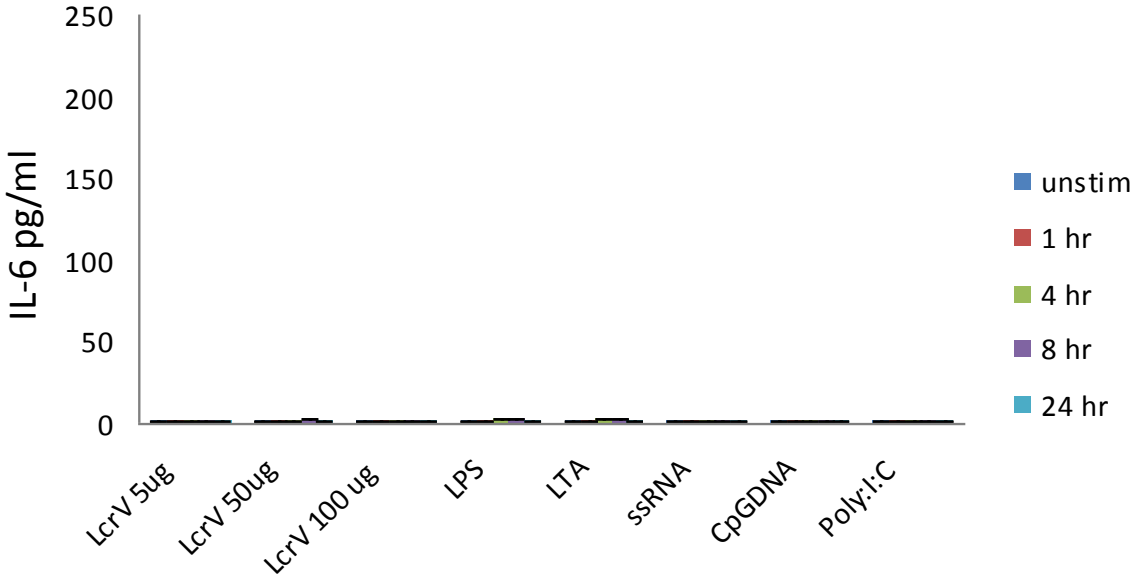


**Figure 5.8 – Secretion of IL-6 in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

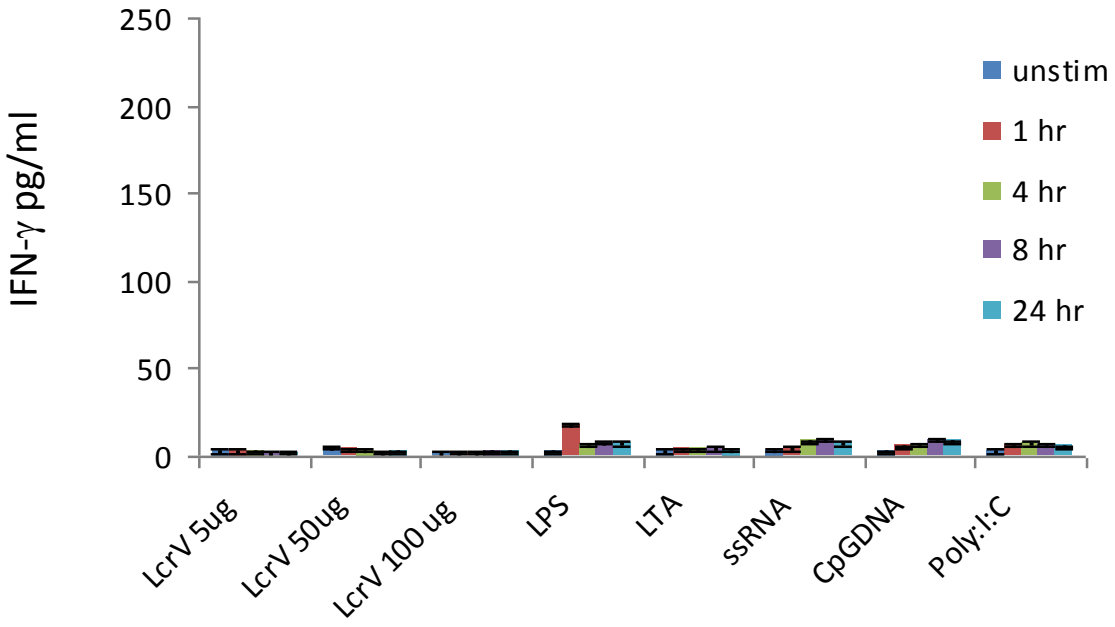


**Figure 5.9 – Secretion of IFN-γ in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

Finally the secretion of IL-10 in response to V antigen as well as PAMPs was investigated. There was no significant IL-10 secretion in response to V antigen stimulation alone for the first 4 hours. 8 hours after stimulation, however, there was some secretion of IL-10 detected, thus suggesting that V antigen might be triggering the secretion of IL-10 in order to modulate the innate immune response. Interestingly, there was some secretion of IL-10 in response to the different PAMPs, in particular LPS and LTA, over a 24 hour period (Figure 5.10).

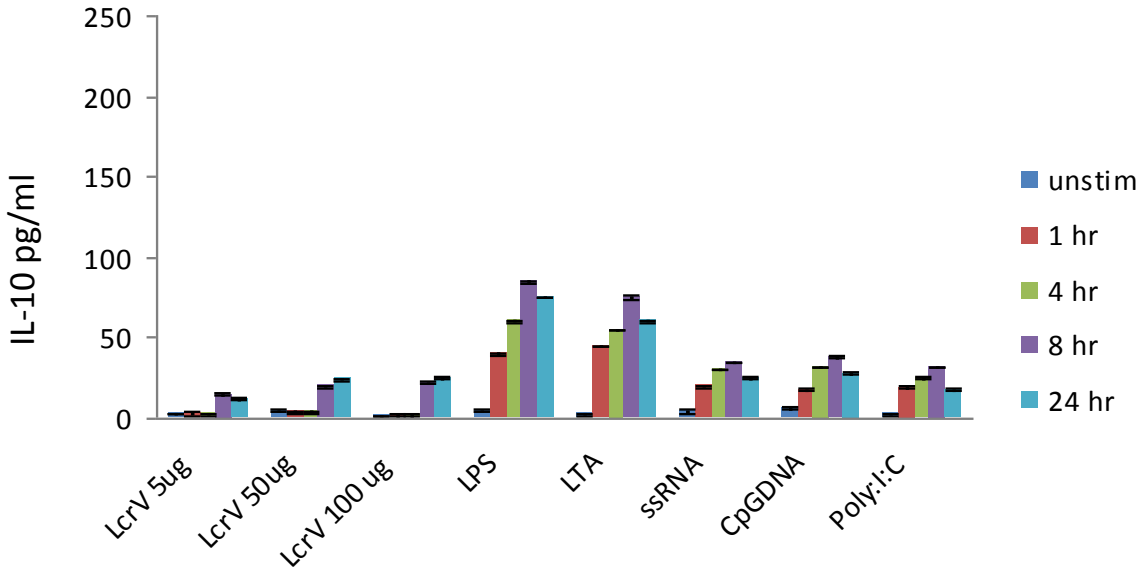


**Figure 5.10 – Secretion of IL-10 in response to V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either V antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

## 5.6. Cytokine secretion in response to pre- or post-treatment with V antigen

In order to determine whether V antigen influenced the cytokine response to known TLR ligands, Mono Mac 6 cells were stimulated with certain PAMPs (LPS, LTA, Poly:I-C, ssRNA and CpG DNA) for 1 hour and either pre-treated or post-treated for 1 hour with V antigen. The presence of cytokines was measured as stated previously.

Initially the effect of pre- and post-treatment of V antigen in the secretion of TNF-α was studied. It was shown that treatment with V antigen prior to PAMP stimulation, inhibited TNF-α secretion (Figure 5.11). When the effect of stimulating the cells with a PAMP first, followed by post-treatment with V antigen, was studied, it was shown that V antigen was still able to inhibit the response. With some PAMPs, post-treatment seemed to be even more effective at inhibiting the TNF-α response compared to pre-treatment.

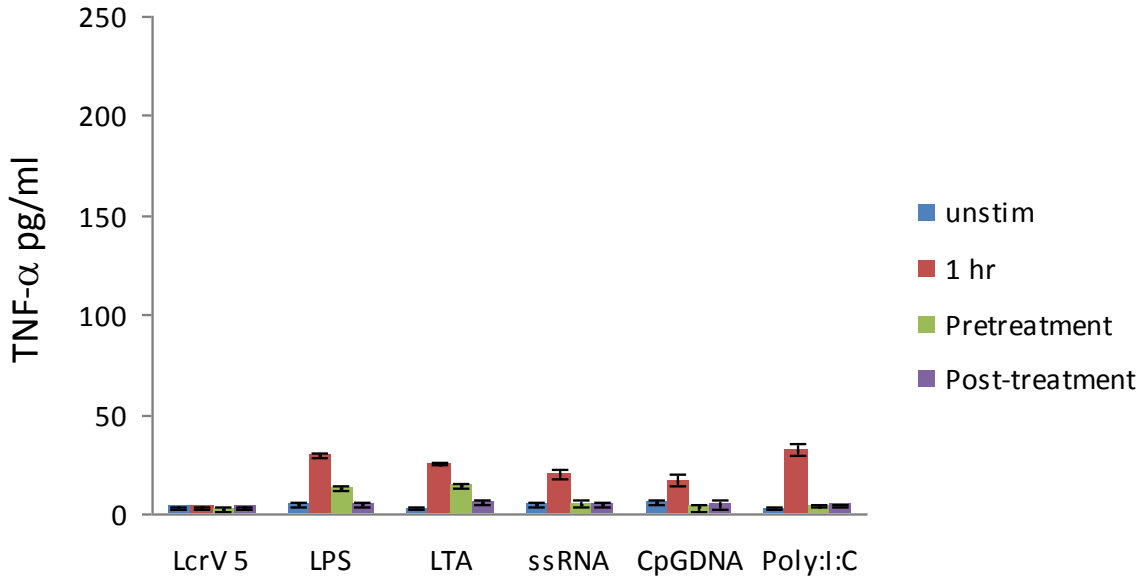


**Figure 5.11 – Secretion of TNF-α in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

When IL-2 (Figure 5.12), IL-4 (Figure 5.13), IL-6 (Figure 5.14) and IFN-γ (Figure 5.15) secretions were tested, all four cytokines were found not to be secreted in response to the different ligands and pre- or post-treatment with V antigen. As

previously mentioned, while this was expected for IL-2, IL-4 and IFN-γ as monocytes do not secrete these cytokines, they are, however, known to secrete IL-6.

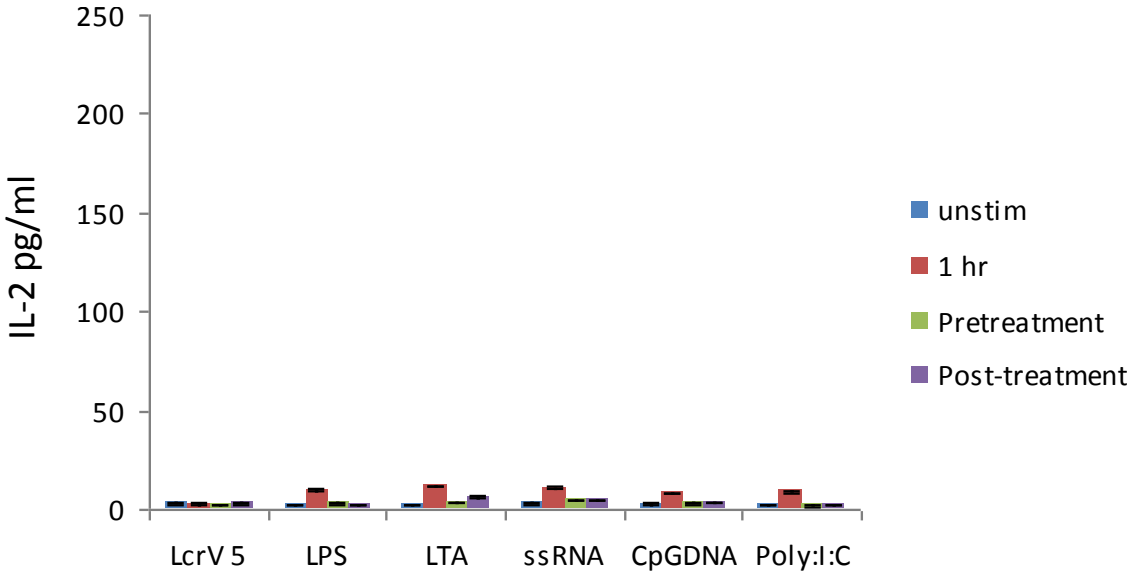


**Figure 5.12 – Secretion of IL-2 in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

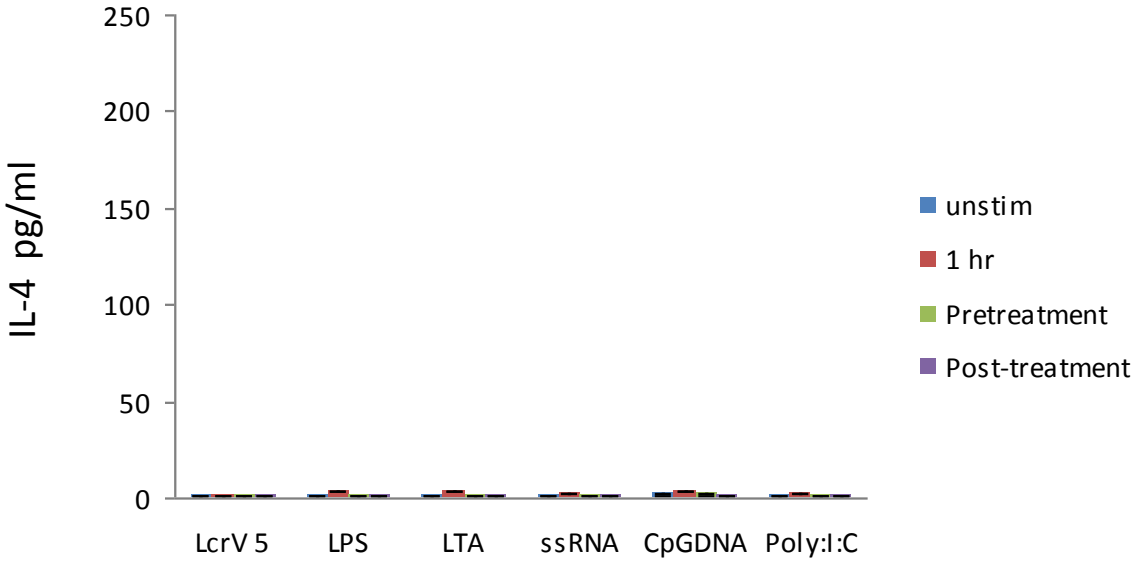


**Figure 5.13 – Secretion of IL-4 in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

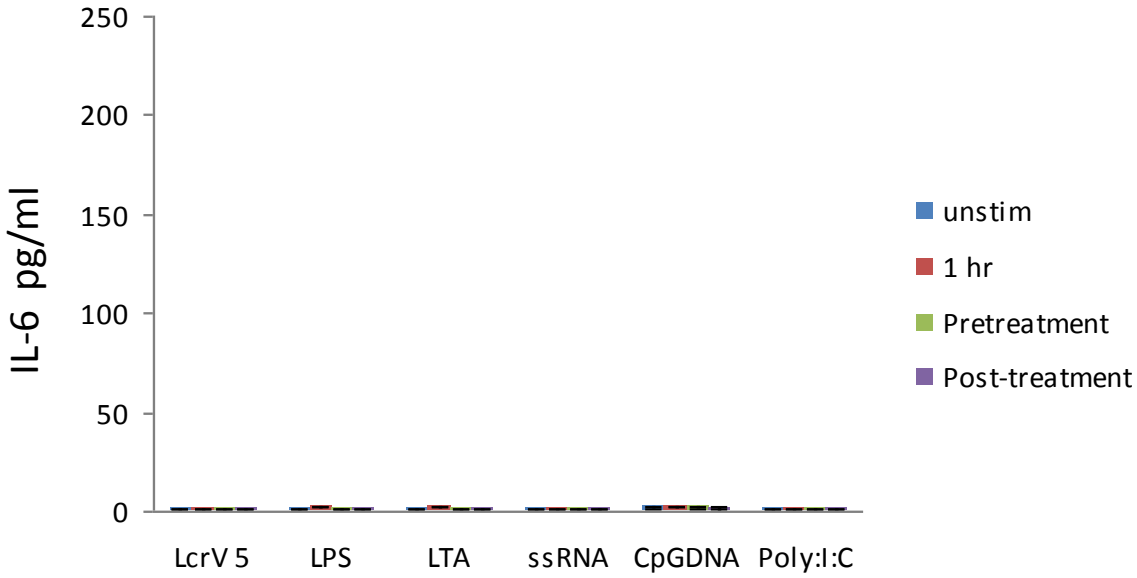


**Figure 5.14 – Secretion of IL-6 in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

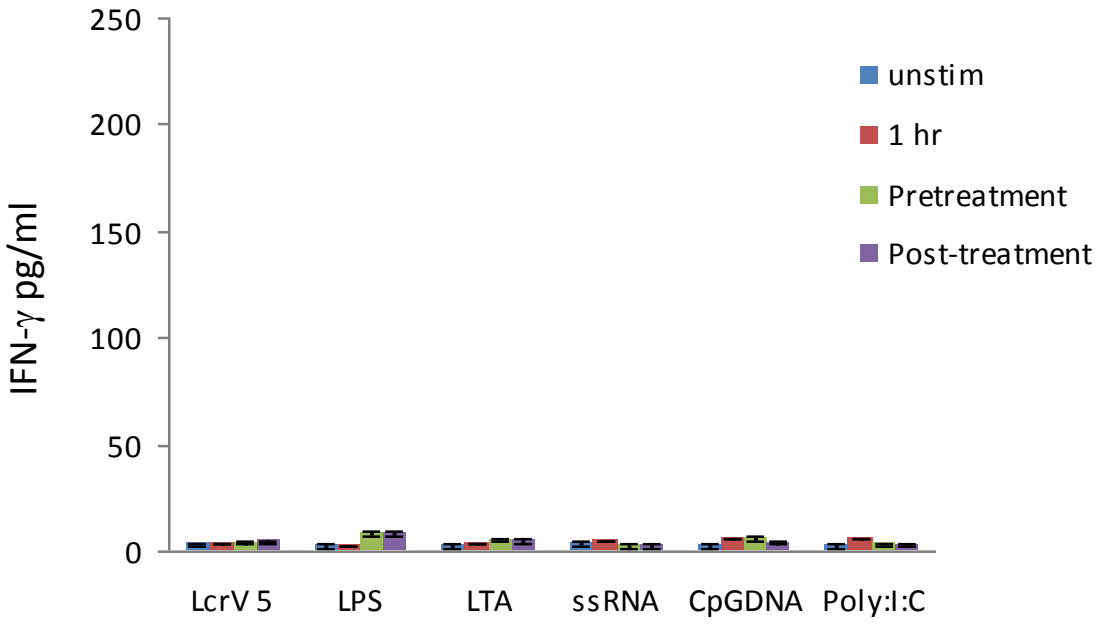


**Figure 5.15 – Secretion of INF-γ in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

Finally, the effect of V antigen pre- and post-treatment in response to PAMPs on IL-10 secretion (Figure 5.16) was investigated. There was significant inhibition of cytokine secretion observed. It would be interesting to investigate in the future the effect of IL-10 over a longer period of time, since it was previously shown to be secreted in higher amounts after 8 hours of incubation.

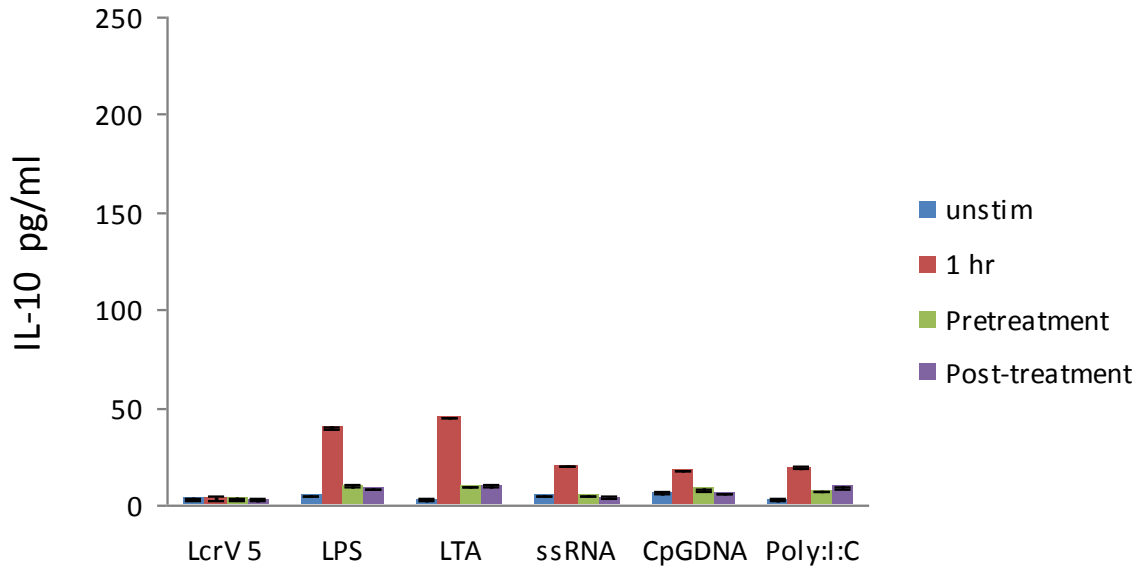


**Figure 5.16 – Secretion of IL-10 in response to pre- and post-treatment with V antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with V antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

## 5.7. Conclusions

In order to determine the effects of V antigen on the innate immune response, NF-κB activation as well as cytokine secretion was investigated in human monocytic cells.

The Mono Mac 6 monocytic cell line was used for these studies. Mono mac 6 is the only cell line to constitutively express phenotypic and functional features of mature monocytes (Ziegler-Heitbrock, et al., 1988). Mono Mac 6 cells have several features of mature blood monocytes such as CD14 antigen expression, phagocytotic ability and the functional ability to produce cytokines. This line is often used as an *in vitro* model to demonstrate the actions of monocytes (Neustock, et al., 1993), which is ideal for the present study.

In this study the activation of NF-κB and cytokine secretion of these cells in response to V antigen as well as different PAMPs was analysed. V antigen was found to activate NF-κB but only at low levels soon after stimulation, with increasing NF-κB being activated throughout the time points collected. V antigen was also found not to be able to stimulate the secretion of TNF-α or IL-6 on its own. Monocytes are not able to produce IL-2, IL-4 or IFN-γ, and our results show this lack of secretion as expected. Monocytes though are able to produce TNF-α and IL-6 and since V antigen was unable to trigger those on its own, it seems that it must antagonise PRRs or have a mechanism of innate immune evasion.

One such mechanism might be the secretion of IL-10, which is known to be an anti-inflammatory cytokine. V antigen was found to be able to stimulate the secretion of IL-10 on its own, thus dampening the innate immune response, although this secretion was observed only eight hours after stimulation. This first observed secretion of IL-10 eight hours after V antigen stimulation also coincides with the strongest activation of NF-κB observed. It is possible that this stronger presence of activated NF-κB might be triggering the production and secretion of IL-10.

When Mono Mac 6 cells were stimulated with different TLR ligands, it was shown that the ligands were able to trigger TNF-α as well as IL-10. Interestingly, when the cells were either pre-treated or post-treated with V antigen, the ligand-induced cytokine response was inhibiting. This inhibiting effect might be, at least in part, due to

the observed suppression of the presence of phosphorilated IκB and, consequently, activated NF-κB. The observed effect of IL-10 secretion also being inhibited by pre- or post-stimulation with V antigen might be a result of this suppression of NF-κB. Therefore, suggesting that V antigen is able to modulate the innate immune response and dampen the signalling cascade and thus the inflammatory response. This might be occurring by first suppressing NF-κB activation and later by the secretion of the anti-inflammatory IL-10.

# *Chapter 6 - Expression Levels of Toll Like Receptors In Human Monocytes In Response to F1 antigen*

## 6.1. *Y. pestis* F1 antigen as an innate immune modulator

Following infection of the mammalian host from the flea, *Y. pestis* is soon phagocytosed and spends a period of intracellular existence in early infection within macrophages. During this intracellular existence, *Y. pestis* develops virulence factors that possess antiphagocytic capabilities. One of these is the F1 antigen, which develops from the outer membrane as long fibers that blend, criss-cross and even knot with each other to create a capsule-like structure protecting the membrane from phagocytosis. These fibres are also easily shed from the bacterium, essentially secreting them into the extracellular medium. Asides from its antiphagocytic ability, F1 has been shown to have an effect in immunoregulation, such as activating macrophages and inducing nitric oxide production, and has been shown to be immunogenic, with titres of anti-F1 immunoglobulin being detected in the serum of mammals, including humans, infected with a F1-positive *Y. pestis* strain.

## 6.2. Effect of F1 on TLR expression

In order to determine the effect that *Y. pestis* F1 antigen has on the expression levels of several TLRs, Mono Mac 6 cells were stimulated with three different concentrations of F1 antigen (5 µg, 50 µg and 100 µg) and collected at certain timepoints over a 24-hour period (1h, 4h, 8h and 24h). The expression levels of the receptors were determined using flow cytometry. The expression levels of CD36 were also determined as it is a receptor different from the TLRs but is also known to associate sometimes with TLR2. The expression levels of these receptors of unstimulated Mono Mac 6 cells were used as a baseline control of Mono Mac 6 receptor expression levels prior to stimulation.

Initially, Mono Mac 6 cells were stimulated with 5 µg of F1 antigen (Figure 6.1). It was shown that all the TLRs, with the exception of TLR3, were downregulated in response to F1 stimulation. F1 antigen seemed to induce downregulation to all TLRs, except TLR3, within the first hour of stimulation and, in contrast to V antigen, F1 seems to be able to sustain low levels of TLR expression for the duration of the 24 hour observation period. TLR3 was initially upregulated but following 4 hours of stimulation with F1 antigen its expression was also downregulated and found to be lower than the basal level for the rest of the 24 hours.

In contrast to TLRs, CD36 was shown to be upregulated in response to F1 antigen. This upregulation occurred 1 hour after stimulation with F1 antigen and by 8 hours the expression level seemed to drop, but rose again throughout the rest of the 24 hour period.

Therefore it seems that in addition to V antigen, F1 seems to modulate PRR receptor expression and in this way manipulates the innate immune response. In the case of TLRs, it seems to downregulate, suggesting that it “dampens” or inhibits the response. Surprisingly, TLR3 seems to be, initially, the only TLR that is unaffected by F1 antigen. It is possible that since TLR3 signals differently compared to the other TLRs that somehow it remains unaffected. F1 antigen could possibly be targeting the MyD88-dependent signalling pathway and since TLR3 is signalling via the MyD88-independent pathway it is not targeted. In contrast to the TLRs, CD36 seems to augment

its expression early on in the infection, possibly in an attempt to promote the internalization of *Y. pestis*, since CD36 is a scavenger receptor.

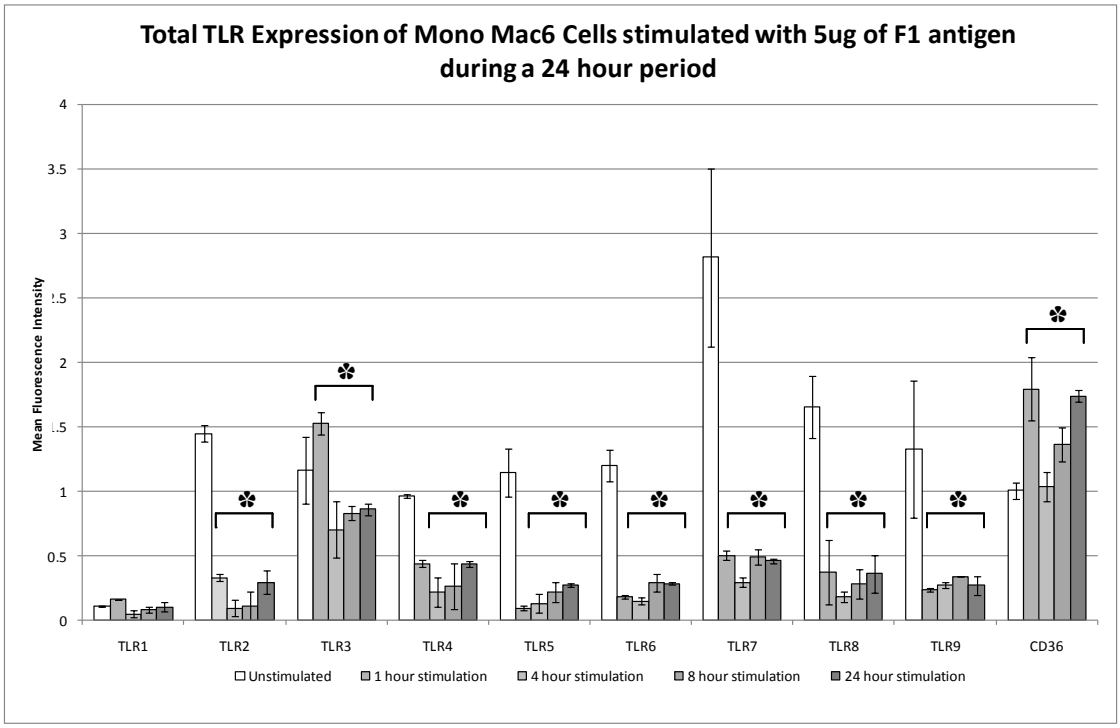


**Figure 6.1 – PRR expression levels in response to 5 µg of F1 antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 on Mono Mac 6 cells stimulated with 5 µg of F1 antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of Mono Mac 6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) difference in expression in comparison to unstimulated cells.

## 6.3. Effect of different concentrations of F1 on TLR expression

In order to test whether the effect of F1 antigen on TLR expression is concentration-dependent, the effect of higher concentrations of F1 antigen 50 µg (Figure 6.2) and 100 µg (Figure 6.3) on Mono Mac 6 cells was investigated.

It was shown that higher concentrations of F1 antigen, 50 µg and 100 µg, had the same effect on TLR and CD36 expression. The same trend of downregulation of TLRs was observed in all TLRs except TLR3, whereas in the case of CD36 there was upregulation (Figure 6.2 and 6.3). The only noticeable differences when the cells were stimulated with 50 µg of F1 antigen was that the overall expression level of all PRRs was reduced to a greater extent compared to 100 µg but also that in any of these two higher doses, TLR3 appears to be more upregulated than downregulated, and intensity of upregulation of both TLR3 and CD36 appears to be dose-dependent, with the higher the dose, the higher the expression levels observed.

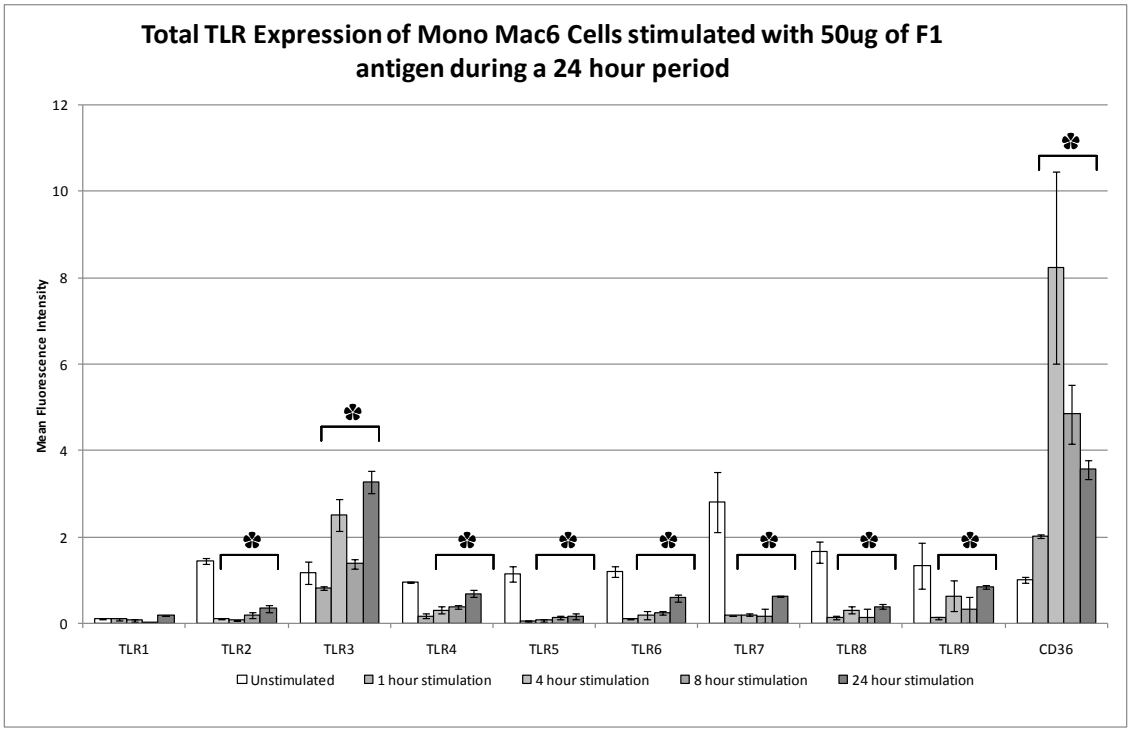


**Figure 6.2 – PRR expression levels in response to 50 µg of F1 antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 of Mono Mac 6 cells stimulated with 50 µg of F1 antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of Mono Mac 6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) difference in expression in comparison to unstimulated cells.

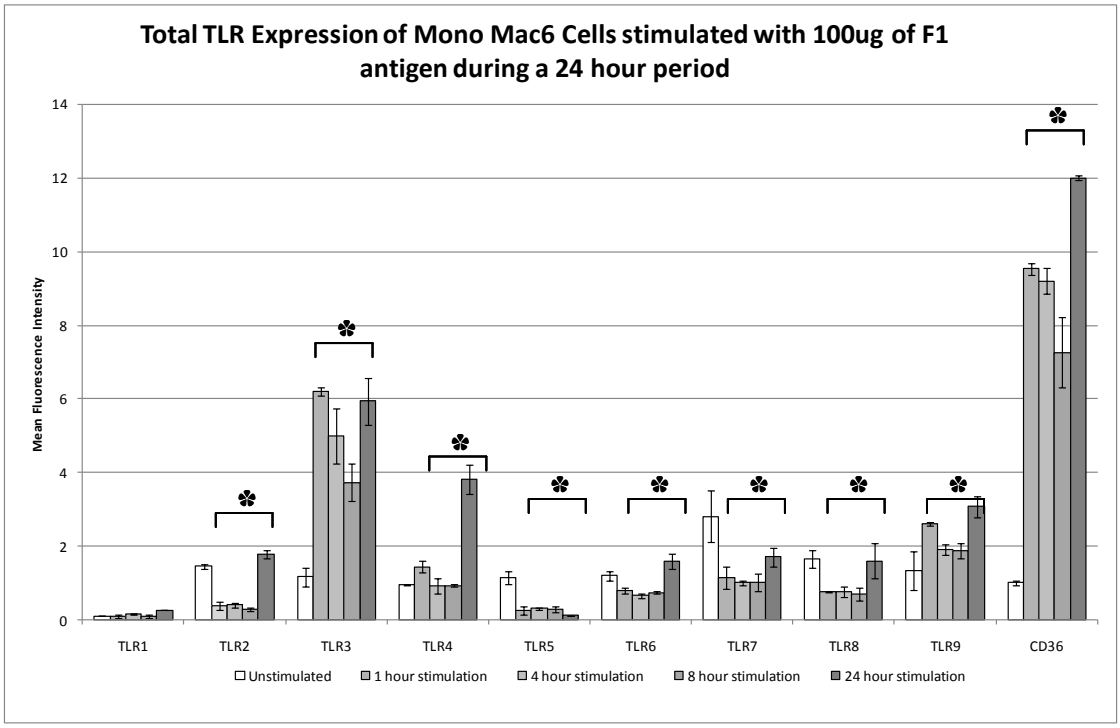


**Figure 6.3 – PRR expression levels in response to 100 µg of F1 antigen.** Receptor expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and CD36 of Mono Mac 6 cells stimulated with 100 µg of F1 antigen for 1 hour, 4 hours, 8 hours and 24 hours with an unstimulated set of Mono Mac 6 cells used as the 0 hour control. The mean fluorescent intensity was obtained from 10,000 cells and was used as a measure of expression of the receptors. Values of respective negative controls have been subtracted from the values originally obtained from the FACSCalibur™. Data represents mean ± stdev. Asterisks indicates statistically significant ($p < 0.05$) upregulation of receptor expression in comparison to unstimulated cells.

## 6.4. Conclusions

Although, several studies have previously shown the immunomodulatory effects of V antigen, this is the first study to examine in-depth the immunodulatory effects of F1.

The effects of F1 antigen on TLR expression were investigated using different concentrations of F1 over a 24-hour period. . Similarly to V antigen, it was shown that F1 antigen does have immunomodulatory effects of PRR expression. F1 induced a sustained downregulation of all TLRs within 1 hour of stimulation, suggesting that *Y. pestis* uses F1 antigen in order to "dampen" the innate immune response in the early stages of the infection. This "dampening" effect also appears to be quite stronger than the one caused by V antigen.

The only TLR that seemed not to be affected by the observed "dampening" effect was TLR3, which underwent upregulation in response to F1 stimulation. TLR3 is the only TLR that does not trigger the MyD88-dependent pathway and thus utilises a different adaptor molecule in order to trigger signalling than any of the other TLRs. F1 might be targeting the MyD88-dependent signalling cascade, modulating the activation of most TLRs, except TLR3 which is the only TLR that becomes unaffected. In the absence of the other TLRs, TLR3 attempts to compensate and triggers the MyD88-independent pathway in order to protect the cell from the infection.

Similarly to TLR3, CD36 expression was also upregulated in response to F1 stimulation. CD36 is a scavenger receptor that helps bind and internalise pathogens. The upregulation observed might possibly be part of a mechanism of trying to clear the bacteria found outside the cells. It might be an attempt to internalise *Y. pestis* within the cells in order to degrade them. And much as observed with cells stimulated with V antigen, the pattern of CD36 expression levels observed is very similar to that of TLR3, suggesting, perhaps, a link between the two receptors or, at least, in the way they are affected by F1 antigen stimulation.

# *Chapter 7 - NF-κB Activation and Cytokine Secretions In Human Monocytic Cells In Response to F1 antigen*

## 7.1. Activation of the NF-κB signalling cascade

As previously mentioned, one of the consequences of activation of TLRs is the expression of genes via NF-κB which, normally, is sequestered by unphosphorylated IκB. One of the most common signalling pathways from TLR activation leads to the phosphorylation of IκB and therefore release of NF-κB. To verify whether this occurs and, therefore, whether cytokine secretion in this instance was linked to NF-κB activation, Mono Mac 6 cells were stimulated in serum-free medium with different concentrations (5 µg, 50 µg and 100 µg) of *Y. pestis* F1 antigen and collected over a 24-hour period (1h, 4h, 8h and 24h).

Furthermore, in order to determine whether F1 antigen influenced the cytokine response to known TLR ligands, Mono Mac 6 cells were also stimulated in serum-free medium with certain PAMPs (LPS, LTA, Poly:I-C, ssRNA and CpG DNA) for 1 hour as well as pre- and post-treated with 5 µg of F1 antigen. These were also collected, the supernatant frozen and stored for cytokine assays while the cells were lysed and used to verify the presence of phospho-IκB.

## 7.2. Presence of phosphorylated IκB in response to F1 antigen

In order to determine whether *Y. pestis* F1 antigen leads to NF-κB activation, cells were stimulated with F1 antigen and lysed at different time points (1h, 4h, 8h and 24h). The protein complex NF-κB is a transcription factor that is involved in the cellular response to stress and, of particular interest to this work, in the activation of the production of cytokines. The presence of IκB in the cell lysate indicates that the transcription of proinflammatory proteins. Western blotting was utilised in order to determine the presence of the phosphorylated form of IκB.

Initially, whether F1 antigen itself can trigger NF-κB activation was investigated. Cell lysates that were only stimulated with F1 antigen at different time points (Figure 7.1) were analysed. It was shown that F1 antigen could trigger some NF-κB activation on its own.

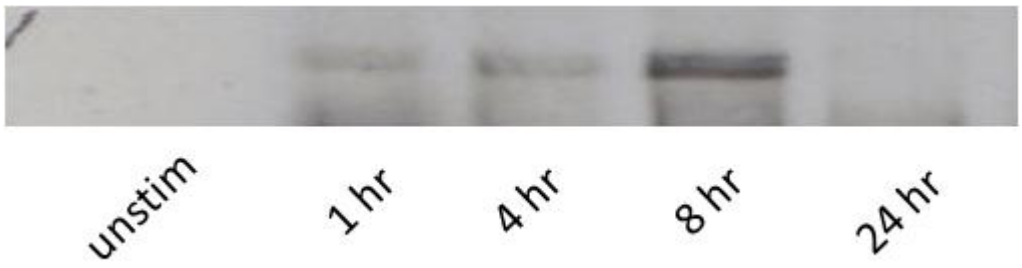


**Figure 7.1 – Presence of phosphorylated IκB in cells stimulated with F1 antigen at different time points.** Mono Mac 6 cells were stimulated with 5 µg of F1 antigen and lysed at 0, 1, 4, 8 and 24 hours. Cell lysates were analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 7.3. Presence of phosphorylated IκB in response to PAMPs

Since F1 antigen has already been shown to be able to modulate TLR expression, it was suggested that it could possibly modulate innate immune responses. In order to test this hypothesis, whether F1 antigen is able to modulate NF-kB activation in response to TLR ligands or PAMPs was investigated.

Initially NF-κB activation in response to PAMPs was investigated. It was shown that there was a strong band corresponding to the phosphorylated form of IkB in response to LPS and LTA and to a lesser extent in response to ssRNA, CpG DNA and Poly:I:C (Figure 7.2).


**Figure 7.2 – Presence of phosphorylated IκB in cells stimulated with PAMPs.** Mono Mac 6 cells were stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for 1 hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 7.4. Presence of phosphorylated IκB in response to PAMPs pre- and post-treated with F1 antigen

Once it was established that the different PAMPs could trigger the NF-κB signalling cascade, whether F1 antigen was able to modulate this response was investigated.

Initially, the effect that pre-treatment with F1 antigen will have on NF-κB activation was investigated. It was shown that the addition of F1 antigen inhibited the activation in response to the PAMPs although there was still some activation in response to the various PAMPs (Figure 7.3). Similar results were obtained when the cells were first stimulated with the PAMPs and subsequently F1 antigen was added (post-stimulation) except that activation of NF-κB was completely inhibited with the cells stimulated first with ssRNA, CpG DNA and Poly:I-C (Figure 7.4).

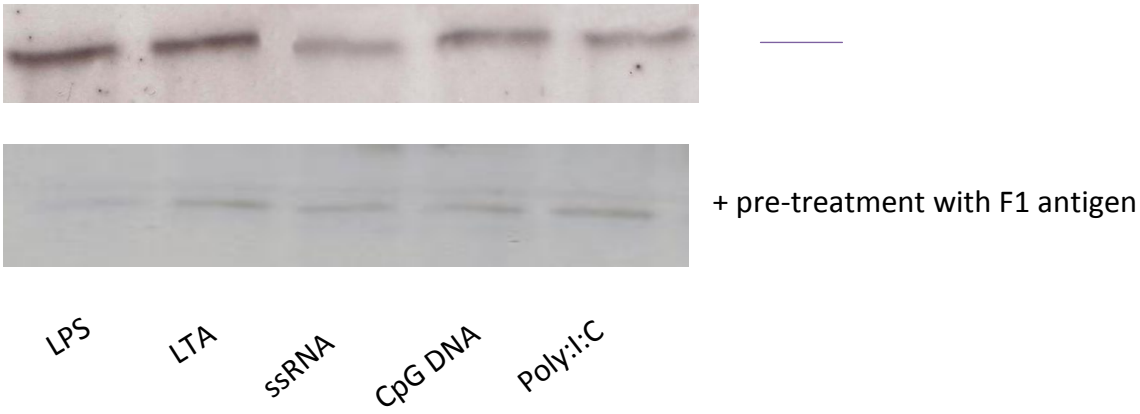


**Figure 7.3 – Presence of phosphorylated IκB in cells pre-treated with F1 antigen and subsequently incubated with PAMPs.** Mono Mac 6 cells were pre-treated with 5 µg of F1 antigen for 1 hour and subsequently stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for a further hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

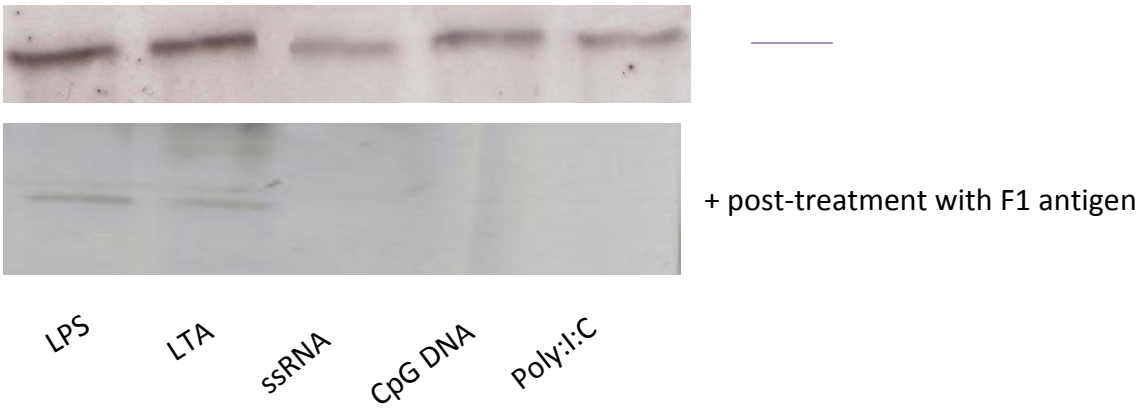


**Figure 7.4 – Presence of phosphorylated IκB in cells post-treated with F1 antigen.** Mono Mac 6 cells were stimulated with either 100 ng/ml LPS, 10 µg/ml LTA, 5 µg/ml ssRNA, 5 pmol CpG DNA or 20 µg/ml Poly:I:C for 1 hour and subsequently post-treated with 5 µg of F1 antigen for a further hour. The cells were lysed and analysed by SDS-PAGE, followed by western blotting with an antibody specific for the phosphorylated form of IκB and the appropriate secondary antibody conjugated to HRP. ECL was used for detection.

## 7.5. Cytokines secreted by cells during a 24-hour period

The supernatants collected from the Mono Mac 6 cells stimulated with different concentrations (5 µg, 50 µg and 100 µg) of *Y. pestis* F1 antigen and collected at certain timepoints within a 24-hour period (1h, 4h, 8h and 24h) were used to measure their cytokine concentrations. The presence of cytokines was measured using a Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II, which is capable of detecting TNF-α, INF-γ, IL-2, IL-4, IL-6 and IL-10, and a FACSCalibur™ to measure the fluorescence from the beads and determine cytokine concentration.

Initially TNF-α secretion in response to different concentrations of F1 antigen (5, 50 and 100 µg) was investigated as well as different PAMPs. It was shown that F1 antigen on its own did not stimulate any TNF-α secretion at any concentration tested. In contrast, the PAMPs, in particular LPS and LTA, stimulated a significant TNF-α response over a 24 hour period, with a peak at 8 hours following stimulation for most PAMPs except for ssRNA, which had its peak at 4 hours, and CpG DNA, which had its peak at 24 hours (Figure 7.5).

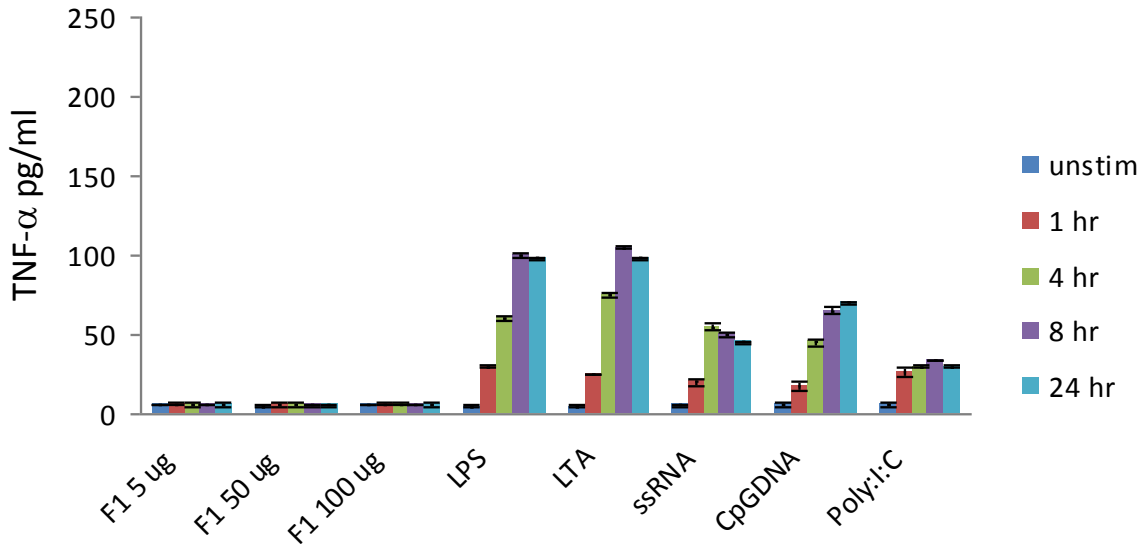


**Figure 7.5 – Secretion of TNF-α in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

In addition, IL-2 (Figure 7.6), IL-4 (Figure 7.7), IL-6 (Figure 7.8) and IFN-γ (Figure 7.9) secretion was also tested. The IL-2, IL-4 and IFN-γ cytokines were found not to be secreted in response to the different ligands, as expected, since monocytes do not secrete these cytokines. Secretion of IL-6 occurred in response to all the ligands used except for F1 antigen, which seemed not to provoke an IL-6 secretion response from the cells.

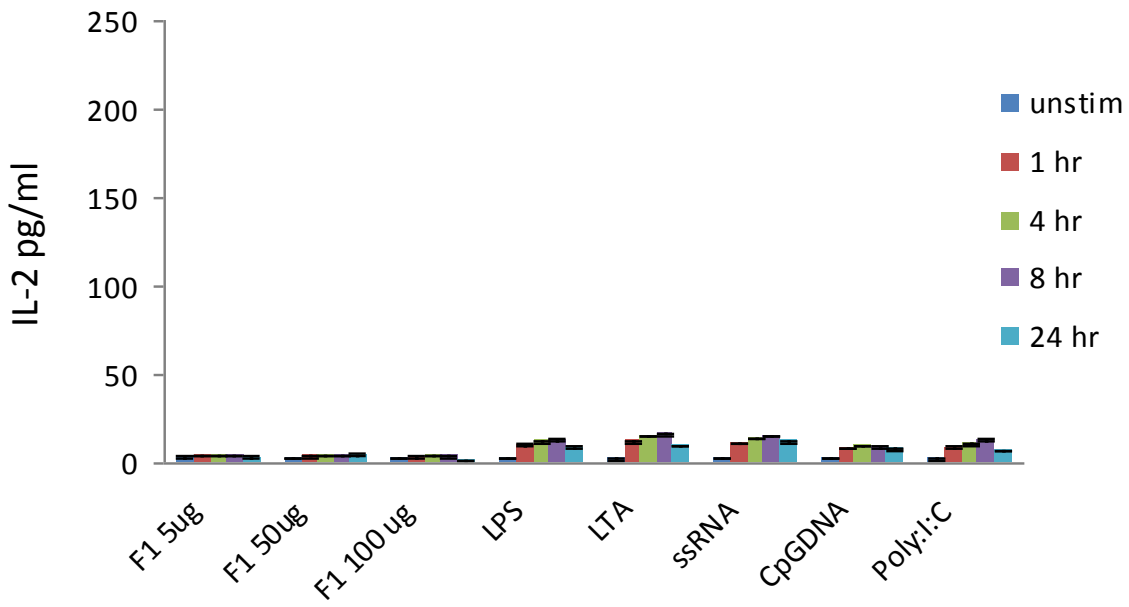


**Figure 7.6 – Secretion of IL-2 in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

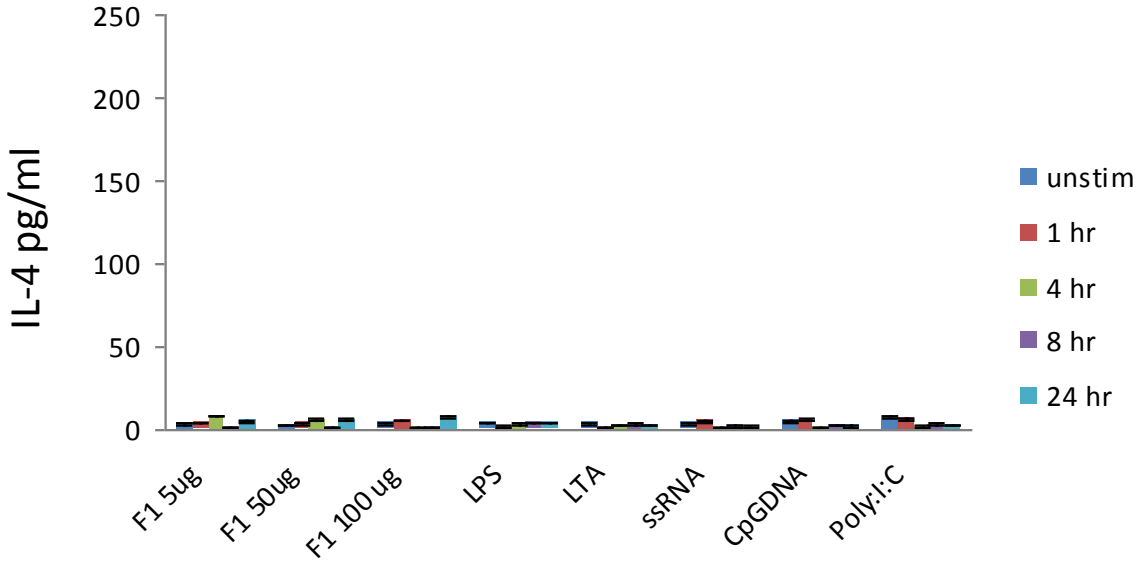


**Figure 7.7 – Secretion of IL-4 in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

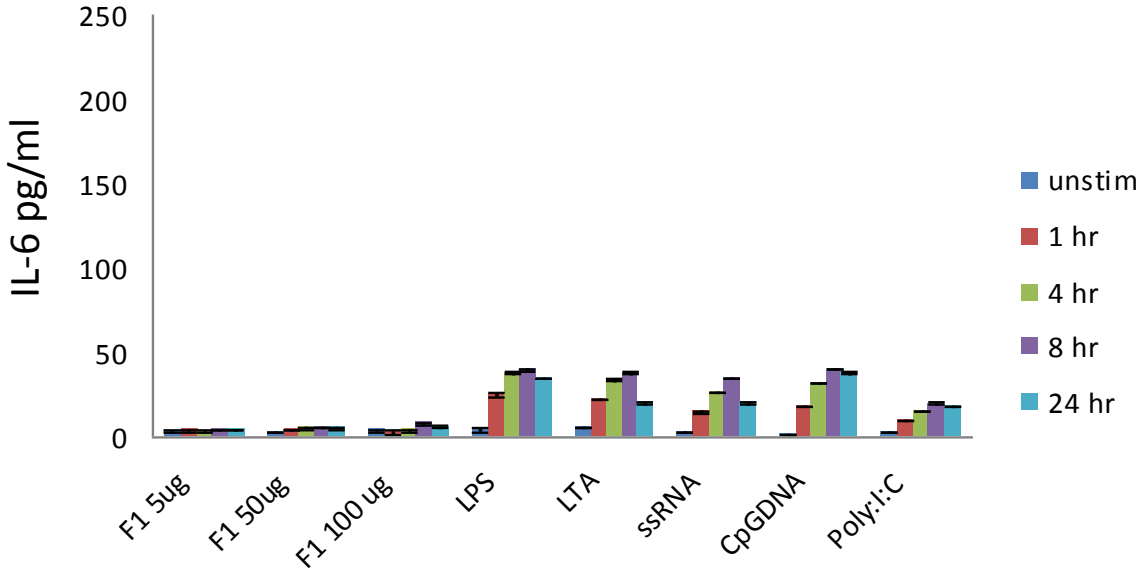


**Figure 7.8 – Secretion of IL-6 in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

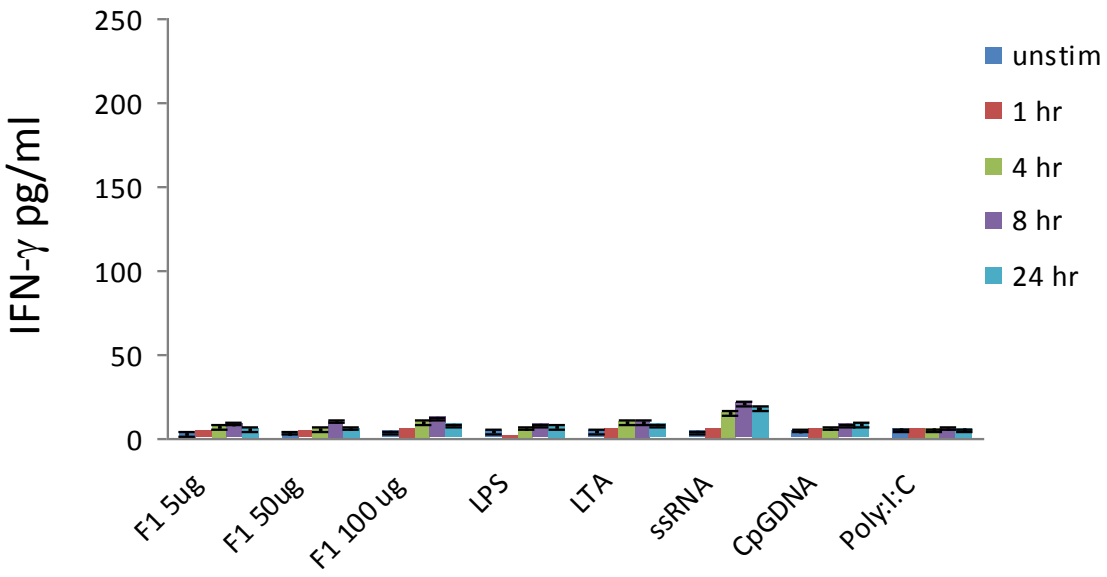


**Figure 7.9 – Secretion of IFN-γ in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

Finally the secretion of IL-10 in response to F1 antigen as well as PAMPs was investigated. There was no significant IL-10 secretion in response to F1 antigen on its own for the first 4 hours, but after 8 hours there was considerable dose-dependent secretion of IL-10 in response to F1 antigen on its own, with the higher the dose of F1, the higher secretion of IL-10, thus suggesting that F1 antigen, like V antigen, might be triggering the secretion of IL-10 in order to modulate the innate immune response. Interestingly, there was considerable secretion of IL-10 in response to the different PAMPs over a 24 hour period (Figure 7.10). Similarly to the other cytokines, there was a peak secretion at 8 hours for the PAMP-stimulated cells.

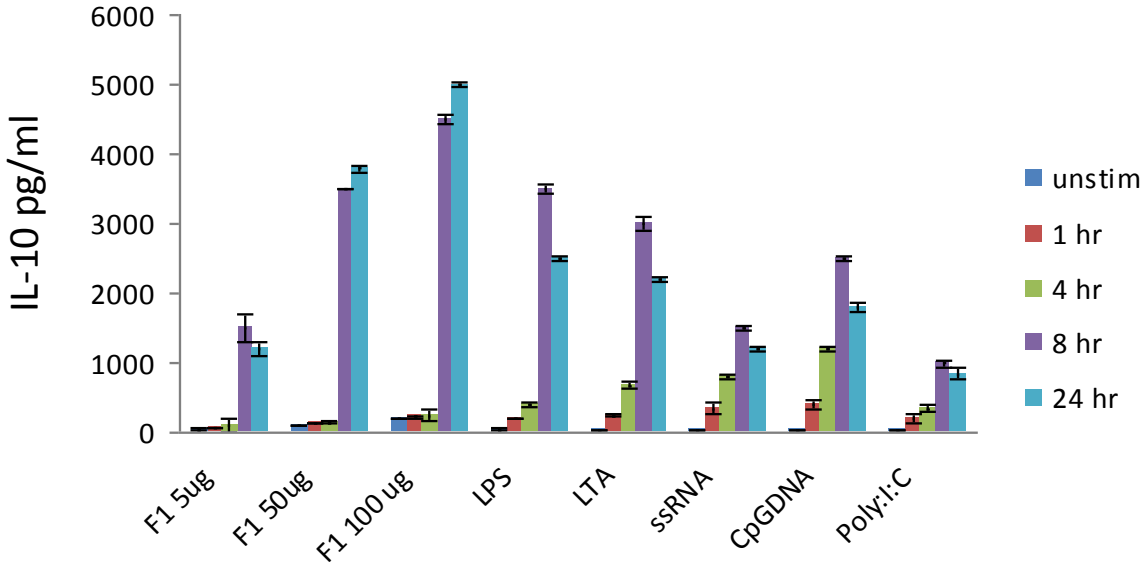


**Figure 7.10 – Secretion of IL-10 in response to F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were stimulated with either F1 antigen or the different PAMPs over a 24 hour period. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

## 7.6. Cytokine secretion in response to pre- or post-treatment with F1 antigen

In order to determine whether F1 antigen influenced the cytokine response to known TLR ligands, Mono Mac 6 cells were stimulated with certain PAMPs (LPS, LTA, Poly:I-C, ssRNA and CpG DNA) for 1 hour and either pre-treated or post-treated for 1 hour with F1 antigen. The presence of cytokines was measured as stated previously.

Initially the effect of pre- and post-treatment of F1 antigen in the secretion of TNF-α was tested. It was shown that treatment with F1 antigen prior to PAMP stimulation, inhibited TNF-α secretion (Figure 7.11). When the effect of stimulating the cells with PAMP first, followed by post-treatment with F1 antigen was tested it was shown that F1 antigen was still able to inhibit the response. In some PAMPs post-treatment seemed to be even more effective at inhibiting the TNF-α response compared to pre-treatment. These results were similar to the effects previously observed with V antigen.

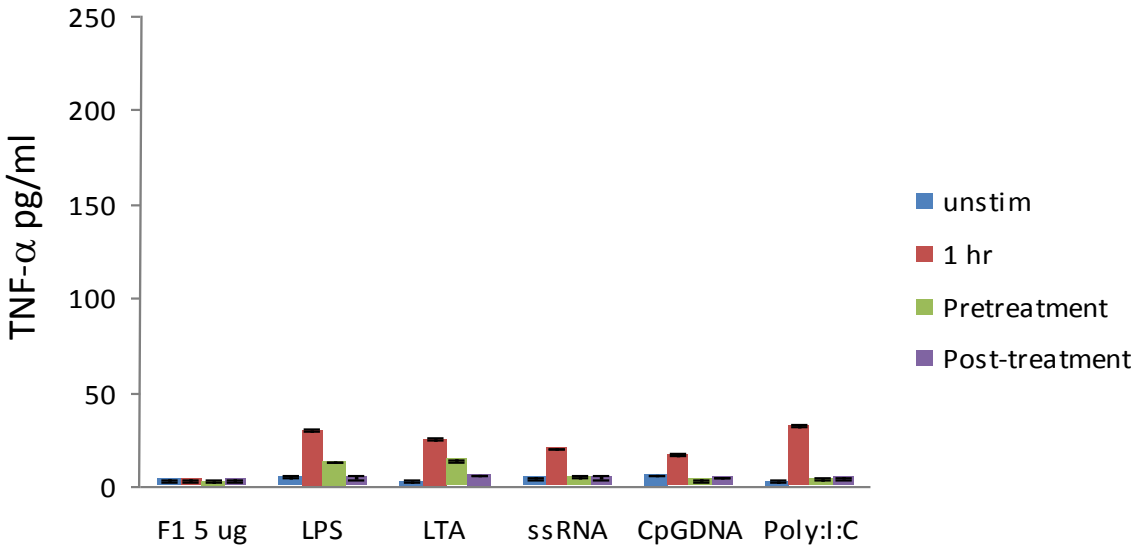


**Figure 7.11 – Secretion of TNF-α in response to pre- and post-treatment with F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with F1 antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson.

When IL-2 (Figure 7.12), IL-4 (Figure 7.13), IL-6 (Figure 7.14) and IFN-γ (Figure 7.15) secretions were tested, IL-2, IL-4 and IFN-γ were found not to be secreted

in response to the different ligands and pre- or post-treatment with F1 antigen as expected. Secretion of IL-6 was inexistent for the cells stimulated only with F1 antigen, showing again the lack of IL-6 secretion in response to F1 antigen as shown previously. Secretion of IL-6 occurred in cells stimulated with any of the other ligands and pre- or post-treatment with F1 antigen showed a clear decrease in secretion compared to the cells stimulated with only one ligand.

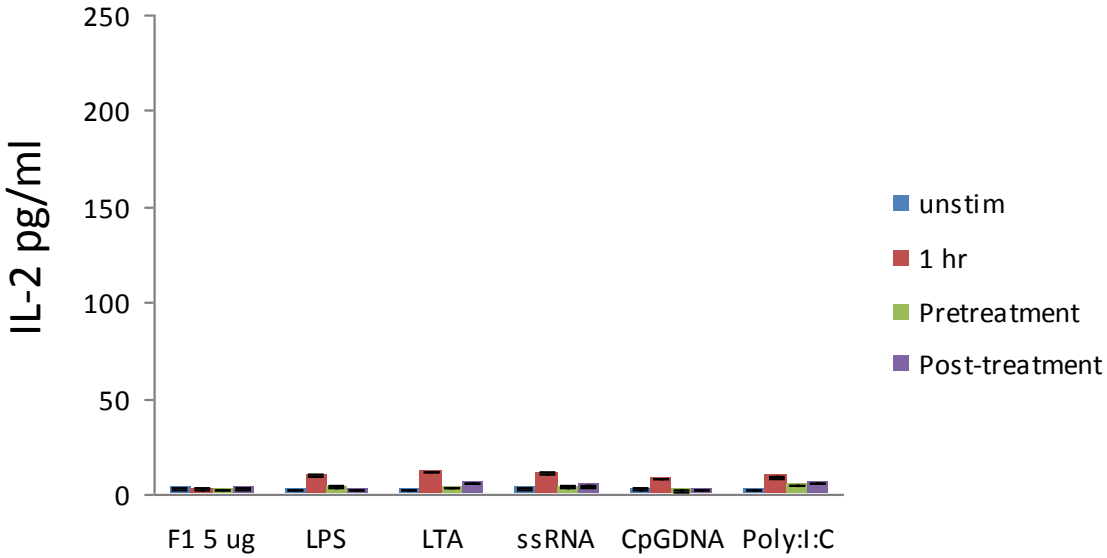


**Figure 7.12 – Secretion of IL-2 in response to pre- and post-treatment with F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with F1 antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

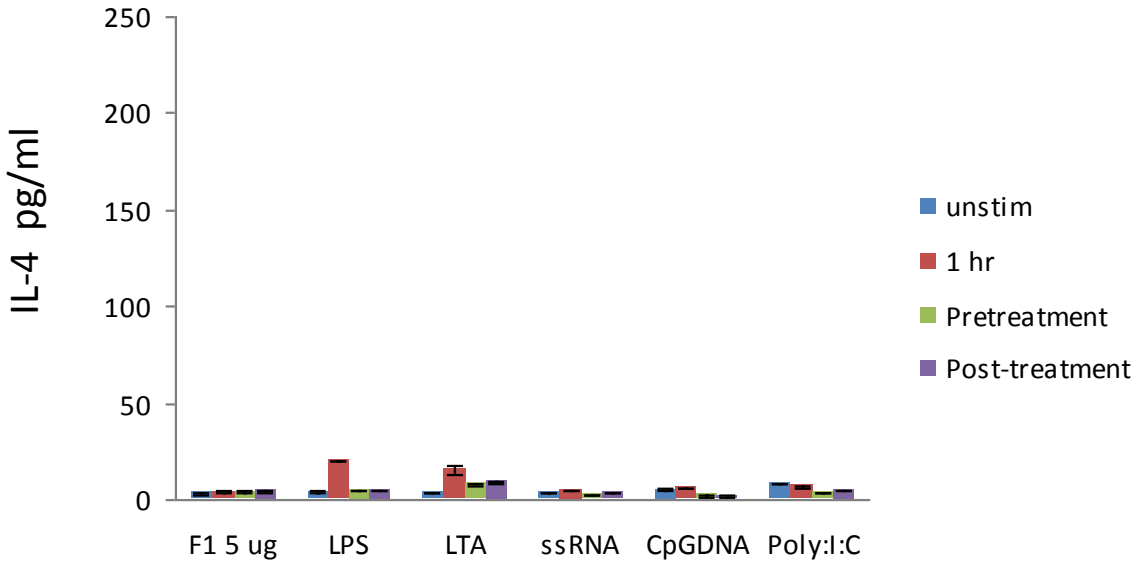


**Figure 7.13 – Secretion of IL-4 in response to pre- and post-treatment with F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with F1 antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

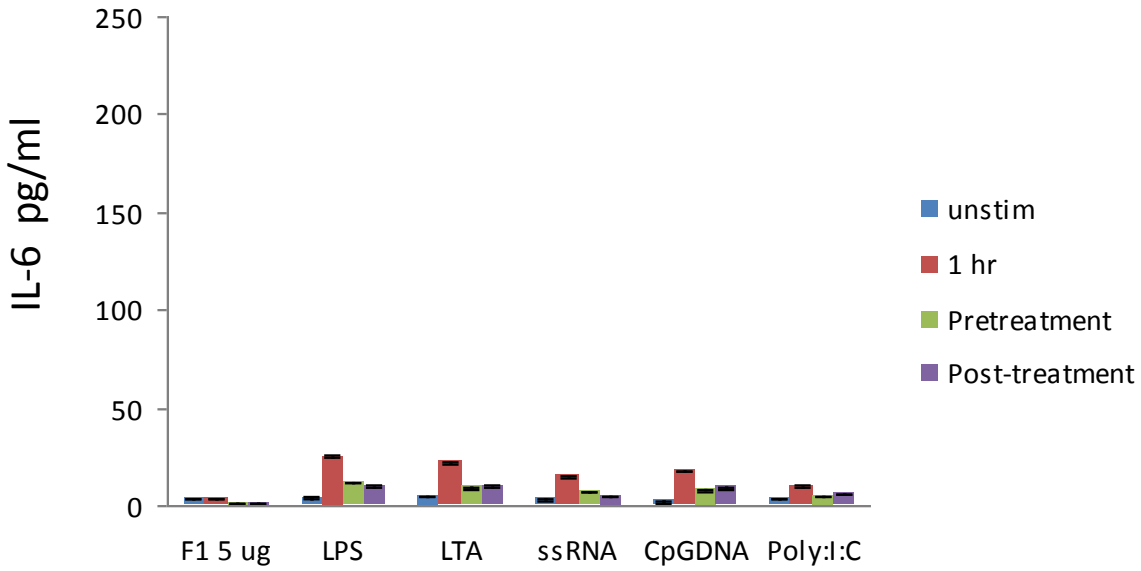


**Figure 7.14 – Secretion of IL-6 in response to pre- and post-treatment with F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with F1 antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3).

Finally, the effect of F1 antigen pre- and post-treatment in response to PAMPs on IL-10 secretion (Figure 7.15) was investigated. Compared to the other cytokines, IL-10 was secreted in higher amounts. There was significant inhibition of cytokine secretion. It would be interesting to investigate in the future the effect of IL-10 over a longer period of time, since it was shown to be secreted in higher amounts after 8 hours of incubation.

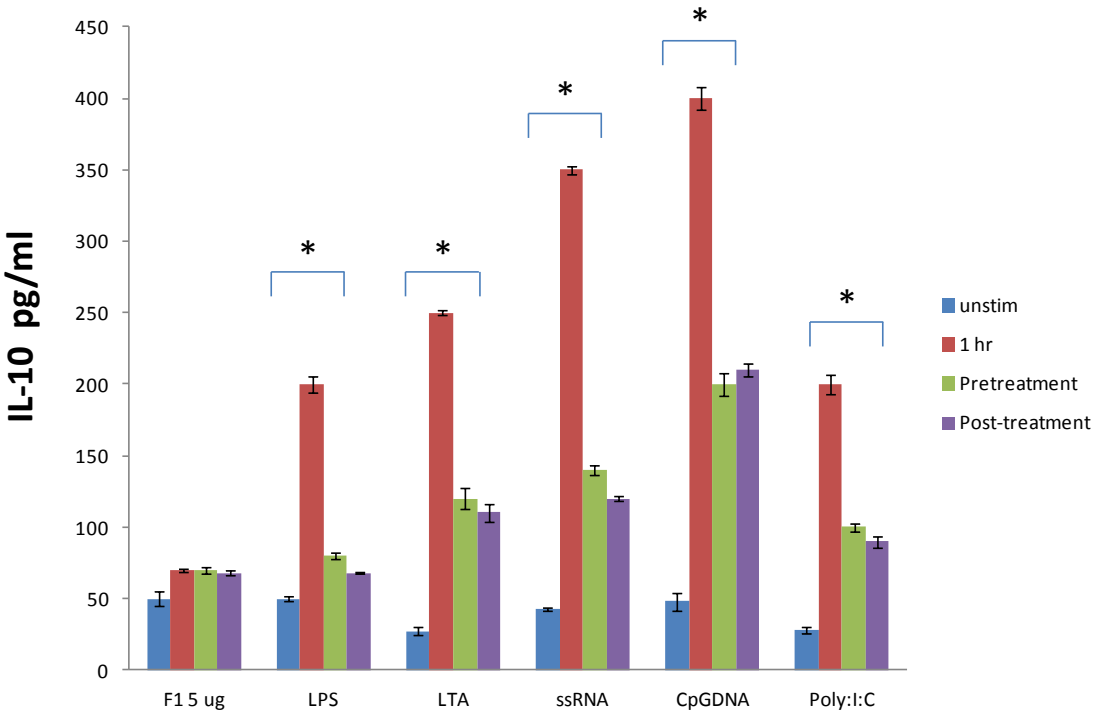


**Figure 7.15 – Secretion of IL-10 in response to pre- and post-treatment with F1 antigen and PAMPS (LPS, LTA, ssRNA, CpG DNA and Poly:I-C).** Human Mono Mac 6 cells were either pre- or post-treated with F1 antigen and different PAMPs. The cytokines were measured in the cell supernatant using the Th1/Th2 cytokine bead array system from Becton Dickinson. Data represents mean ± stdev (n = 3). Asterisks indicates statistically significant ($p < 0.05$) secretion of cytokine in comparison to unstimulated cells.

## 7.7. Conclusions

In order to determine whether F1 could modulate NF-kB activation as well as cytokine secretion a human monocytic cell line was stimulated with F1 antigen. F1 antigen was found to activate NF-κB but only at low levels soon after stimulation, with increasing NF-κB being activated throughout the time points collected up to 8 hours after stimulation with F1. By 24 hours after stimulation, NF-κB activation appeared to be non-existent. TNF-α and IL-6, which are two cytokines secreted by monocytes, were not triggered in response to F1 stimulation – suggesting that F1 must antagonise PPRs and/or inhibit innate immune responses.

One mechanism that F1 might be employing in order to inhibit the innate immune response is the induction of IL-10 secretion. IL-10 is a known anti-inflammatory cytokine and it was found to be secreted by monocytes in response to F1 stimulation, although, much like what was seen in cells stimulated with V antigen, secretion was only observed 8 hours after stimulation with F1. This coincides with the observed maximum activation of NF-κB.

The human monocytic cell line was shown to be able to respond to different PAMPs as there was TNF-α, IL-6 and IL-10 secretion in response to LPS, LTA, ssRNA, CpGDNA and Poly:I:C, therefore demonstrating that the cells were capable of producing these cytokines, but not in response to F1 antigen.

Interestingly, pre- or post-treatment of monocytes with F1 antigen was able to inhibit the cytokine response, suggesting that F1 antigen is able to modulate the innate immune response similarly to V antigen. Its mechanism of action could be downregulation of the PRRs as it was observed earlier, suppression of NF-κB activation, secretion of IL-10 thus inhibiting the inflammatory response or a combination of the above.

# *Chapter 8 - Intracellular Trafficking and Targeting of Exogenous V and F1 Antigens In Human Monocytic Cells*

## 8.1. Introduction

The function of the innate immune response is to recognise invading pathogens and to mount and inflammatory response against them. It employs germ-lined encoded receptors, termed pattern recognition receptors, in order to recognise molecular "signatures" that exist on pathogens but not on the host. Recently it has emerged that the Toll-like receptor (TLR) family of proteins is an integral part of the human innate immune system (Akira, 2001; Medzhitov & Janeway, 2002). TLRs are expressed on immune cells and are able to distinguish a great variety of microbial ligands, such as cell wall components like lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria, bacterial flagellin, CpG DNA, as well as viral DNA or single stranded RNA (Takeda, et al., 2003).

To date at least ten different TLR proteins have been discovered in humans that can recognise a wide variety of microbial conserved patterns (Akira, 2001). All identified TLRs are type I transmembrane proteins, whose intracellular domains contain regions homologous to the intracellular domains of IL-1R and are referred to as TIR domains (Takeda, et al., 2003). These intracellular domains are able to trigger signalling pathways known to activate the nuclear factor kappa B (NF-κB) (Medzhitov, et al., 1998; O'Neill, 2000), which in turn leads to the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8. The membrane distribution of TLRs as well as their intracellular trafficking is only now beginning to be investigated.

Most TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) seem to activate cells by engaging their ligands on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 seem to trigger signalling intracellularly. These latter TLRs have been shown to reside in the ER and to recognise their ligands once they have been endocytosed (Heil, et al., 2003; Nishiya & DeFranco, 2004). TLR4 is the most studied TLR, and has been found to be recruited to lipid rafts upon stimulation by bacterial LPS (Triantafilou, et al., 2002), and to be targeted to the Golgi apparatus (Latz, et al., 2002). This intracellular targeting seems to be independent of signalling. TLR2 has also been found to reside in lipid rafts after stimulation by Gram-positive bacterial products and to be similarly targeted to the Golgi apparatus (Triantafilou, et al., 2004).

The main question that remains is whether V and F1 antigen from *Y. pestis* internalise and where they are being targeted to within the cell. Visual verification of the location of these proteins was sought. Since it has been shown that these antigens interfere with TLR expression and signalling, it is highly likely that they co-localise with TLRs. The localization of TLR2 and TLR4 in response to stimulus by these bacterial proteins was verified. TLR2 was chosen given previous studies showing LcrV binding with this receptor (Sing, et al., 2002; Reithmeier-Rost, et al., 2004; Abramov, et al., 2007) and also being found to reside in lipid rafts after stimulation with Gram-positive bacterial products and targeted to the Golgi apparatus (Triantafilou, et al., 2004). TLR4 was selected as one of the best studied TLRs and also due to being found to reside in lipid rafts after stimulation by LPS (Triantafilou, et al., 2002) and to be targeted to the Golgi apparatus (Latz, et al., 2002).

In addition, an interesting question is whether the internalisation of V and F1 antigens is linked with signalling, since it has been shown that they can modulate TLR expression as well as TLR-induced responses. Given the difference of TLR3 expression in previous results due to stimulation with either V or F1, the only human TLR known to not use the MyD88-dependent pathway, it was decided to determine the localization of MyD88 within the cell in response to stimulus by these bacterial proteins.

Confocal microscopy was utilised in order to determine the intracellular trafficking and targeting of V and F1 antigens and whether their internalization was linked with signalling.

## 8.2. Internalisation of V antigen

Mono Mac 6 cells were incubated with V antigen conjugated to Oregon Green (OG-V) for 30 min. Following stimulation with OG-V the cells were fixed and the endosomes were labelled with an antibody against EEA1, a protein within the early endosomes. In addition, either TLR2 or TLR4 was labelled with a specific antibody and the appropriate secondary antibody conjugated to Cy5. It was shown that V antigen was concentrated in a compartment within the cells, which was distinct from the endosomes (Figure 8.1). TLR2 seemed to co-localise with V antigen, whereas TLR4 seemed to be co-localising with the endosomal marker.

In order to determine whether stimulation with PAMPs, affected the intracellular tracking and targeting of V antigen and/or TLRs, Mono Mac 6 cells were stimulated with either LTA (Figure 8.2, top panels), or LPS (Figure 8.2, lower panels) in the presence of V antigen. The cells were subsequently fixed and labelled for EEA1, the endosomal marker, as well as TLR2 or TLR4. It was shown that in response to LTA, TLR2 strongly co-localised with OG-V in a compartment distinct from the endosomes (Figure 8.2, top panels). Similar results were obtained upon LPS stimulation, where TLR4 seemed to co-localise with OG-V in a compartment within the cells that was not the endosomes (Figure 8.2, lower panels).

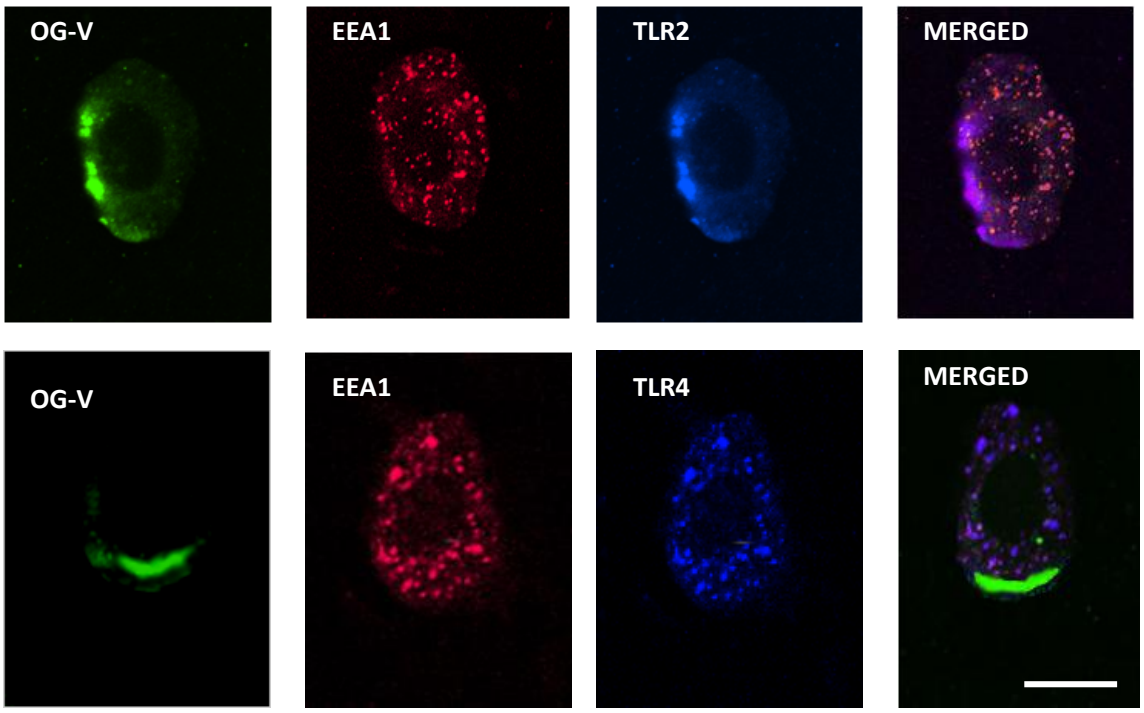


**Figure 8.1 – V antigen localises in an intracellular compartment.** Intracellular distribution of V antigen labelled with Oregon-Green (OG-V) (green), TLR2 (blue) and TLR4 (blue). The endosomes were labelled with antibody against EEA1 (red). Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

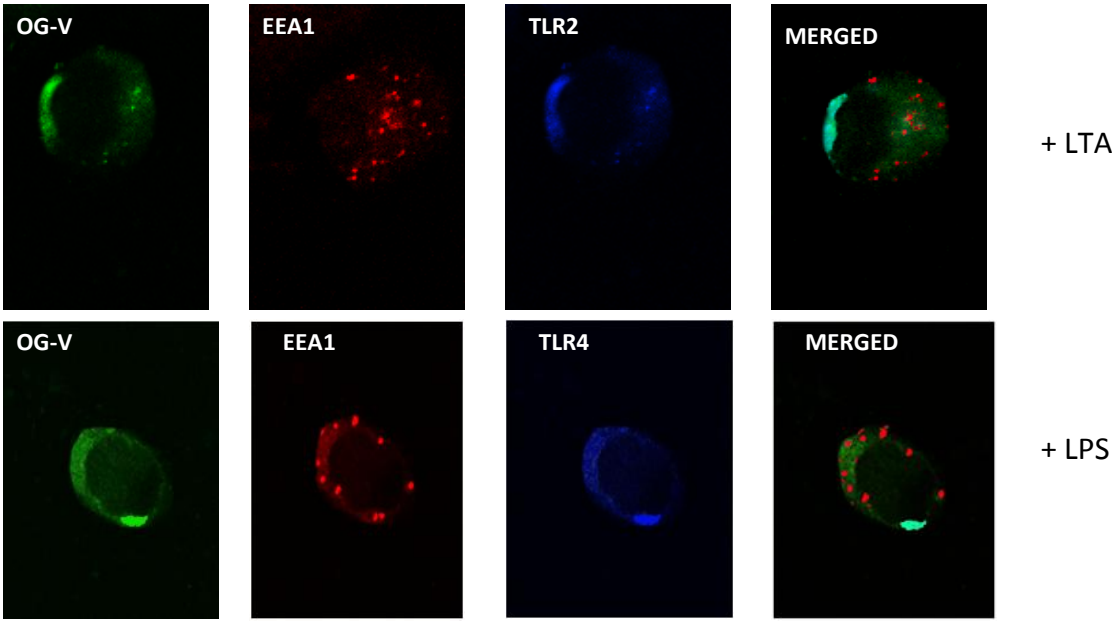


**Figure 8.2 – V antigen localises in an intracellular compartment along with TLRs in response to PAMPs.** Intracellular distribution of V antigen labelled with Oregon-Green (OG-V) (green), TLR2 (blue) and TLR4 (blue) in response to their respective ligands. The endosomes were labelled with antibody against EEA1 (red). Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.3. Intracellular co-localisation of V antigen and TLR4 in the presence and absence of LPS

Since it had already been shown that V antigen co-localises with TLR4 in response to LPS in an intracellular compartment that was distinct from the endosomes, confocal microscopy was utilised in order to determine which intracellular compartment they were concentrating in.

Initially the co-localisation of V antigen with TLR4 in the presence and absence of LPS was investigated. It has previously been shown that in response to LPS, TLR4 is targeted to the Golgi apparatus (Latz, et al., 2002), thus it was investigated whether the Golgi apparatus was the compartment that V antigen and TLR4 were co-localising in.. Mono Mac 6 cells were incubated with V antigen conjugated to Oregon Green (V-OG) for 30 min in the presence or absence of 100 ng/ml LPS. Following stimulation the cells were fixed and the Golgi apparatus was labelled with an antibody against GM130, a protein within the Golgi apparatus, whereas TLR4 was labelled with a TLR4-specific antibody and the appropriate secondary antibody conjugated to Cy5.

Confocal microscopy revealed that in the absence of LPS, V antigen co-localised with the Golgi apparatus and to a lesser extent with TLR4 (Figure 8.3, top panels). TLR4 seemed to be localised in endosomal compartments. When the cells were stimulated with LPS (Figure 8.3, lower panels) V antigen was found to be targeted to the Golgi apparatus, along with TLR4. Thus there was more prominent co-localisation of V antigen and TLR4 in the Golgi apparatus following LPS stimulation. V antigen must interact with TLR4 on the cell surface in response to LPS, and subsequently they must internalise along with TLR4 and be targeted to the Golgi apparatus.

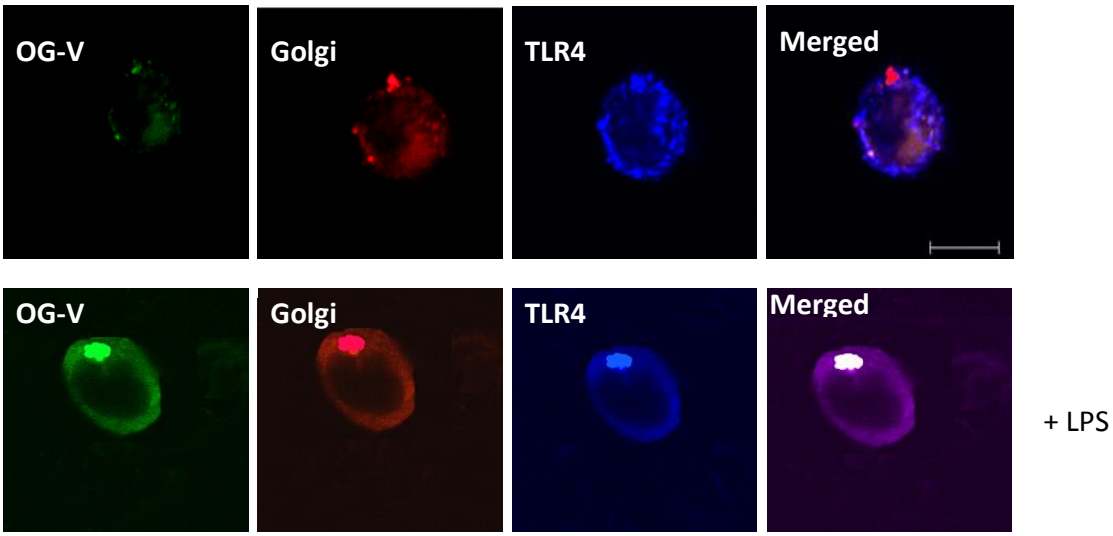


**Figure 8.3 – V antigen and TLR4 co-localise in the Golgi in response to LPS.** Intracellular distribution of V antigen and TLR4. V antigen labelled with Oregon-Green (OG-V) (green) and TLR4 (blue) co-localise within the Golgi apparatus (red) in response to LPS. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.4. Intracellular co-localisation of V antigen and TLR2 in the presence and absence of LTA

Since V antigen was shown to be targeted to the Golgi apparatus along with TLR4 in cells that had been exposed to LPS, the question was raised whether this was also the case with TLR2 in response to LTA.

In the absence of LTA (Figure 8.4, top panels) it was shown that V antigen co-localised in the Golgi apparatus with TLR2. TLR2 became more concentrated within the Golgi apparatus upon stimulation with LTA (Figure 8.4, lower panels), suggesting that similarly to TLR4, V antigen must interact with TLR2 on the cell surface in the presence of LTA and subsequently is targeted to the Golgi apparatus.

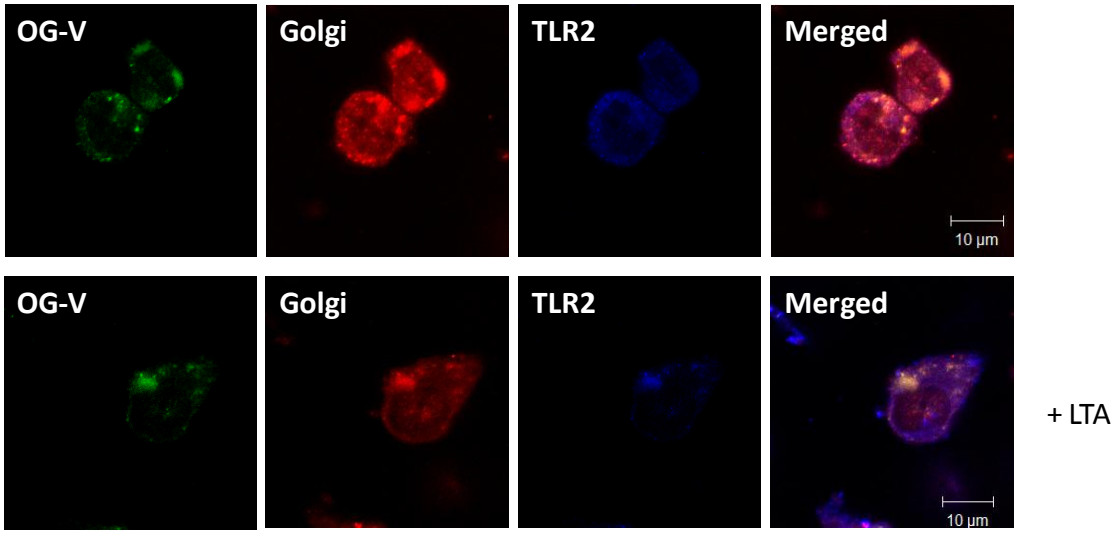


**Figure 8.4 – V antigen and TLR2 co-localise with the Golgi in response to LTA.** Intracellular distribution of V antigen and TLR2 in the presence (lower panels) and absence (top panels) of LTA. V antigen labelled with Oregon-Green (OG-V) (green) and TLR2 (blue) co-localise within the Golgi apparatus (red) in response to LTA. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 µm.

## 8.5. MyD88 activation in the presence and absence of V antigen

Since V antigen had been shown to modulate TLR4 responses, the question that arose was whether it also interfered with TLR2-induced signalling. Mono Mac 6 cells were incubated with either 100 ng/ml LPS (Figure 8.5) or 10 μg/ml LTA (Figure 8.6) for 30 min in the presence or absence of V antigen. Following stimulation, the cells were fixed and the Golgi apparatus was labelled with an antibody against GM130, a protein within the Golgi apparatus, whereas TLR4 and TLR2 were labelled with a TLR-specific antibodies and the appropriate secondary antibody conjugated to Cy5. MyD88, the signalling adaptor molecule for both TLR2 and TLR4, was labelled with an antibody conjugated to FITC.

Confocal microscopy revealed that in the presence of either LPS or LTA, MyD88 co-localised with TLR4 or TLR2 respectively, suggesting that activation had occurred (Figure 8.5 & 8.6, top panels). In contrast, when the cells were stimulated in the presence of V antigen, TLR2 and TLR4 were still targeted to the Golgi apparatus but did not co-localise with the signalling molecule, MyD88 (Figure 8.5 & 8.6, lower panels), therefore suggesting that the presence of V antigen inhibits MyD88 recruitment to TLRs, and thus abrogates signalling.

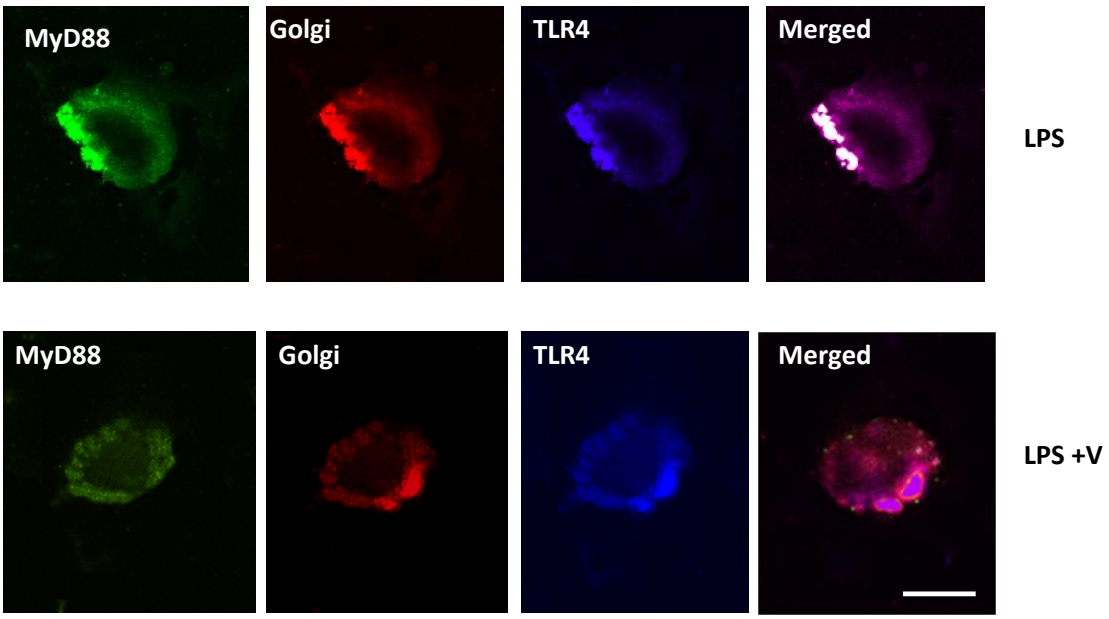


**Figure 8.5 – MyD88 recruitment in the presence and absence of V antigen.** Intracellular distribution of TLR4 and MyD88 in response to LPS (top panels) or LPS in the presence of V antigen (lower panels). TLR4 (blue) co-localised with MyD88 (green) in the Golgi apparatus (red) in response to LPS. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

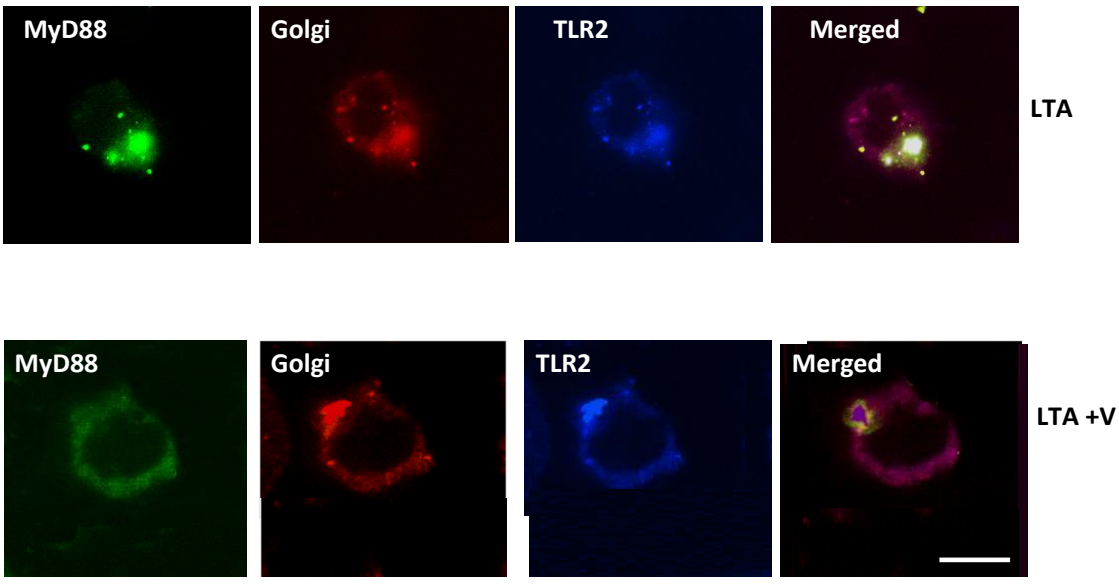


**Figure 8.6 – MyD88 recruitment in the presence and absence of V antigen.** Intracellular distribution of TLR2 and MyD88 in response to LTA (top panels) or LTA in the presence of V antigen (lower panels). TLR2 (blue) co-localised with MyD88 (green) in the Golgi apparatus (red) in response to LTA. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.6. Intracellular co-localisation of F1 antigen and TLR4 in the presence and absence of LPS

Since it had already been shown that V antigen co-localises with TLR4 in response to LPS in Golgi apparatus, confocal microscopy was utilised in order to determine which intracellular compartment F1 antigen was targeted to.

Initially the co-localisation of F1 antigen with TLR4 in the presence and absence of LPS was investigated. Mono Mac 6 cells were incubated with F1 antigen conjugated to Oregon Green (OG-F1) for 30 min in the presence or absence of 100 ng/ml LPS. Following stimulation the cells were fixed and the Golgi apparatus was labelled with an antibody against GM130, a protein within the Golgi apparatus, whereas TLR4 was labelled with a TLR4-specific antibody and the appropriate secondary antibody conjugated to Cy5.

Confocal microscopy revealed that in the absence of LPS, F1 antigen co-localised with the Golgi apparatus but not with TLR4, which appeared to be in the endosomes (Figure 8.7, top panels). When the cells were stimulated with LPS (Figure 8.7, lower panels) F1 antigen was found to be targeted to the Golgi apparatus, along with TLR4. Thus there was more prominent co-localisation of F1 antigen and TLR4 in the Golgi apparatus following LPS stimulation.

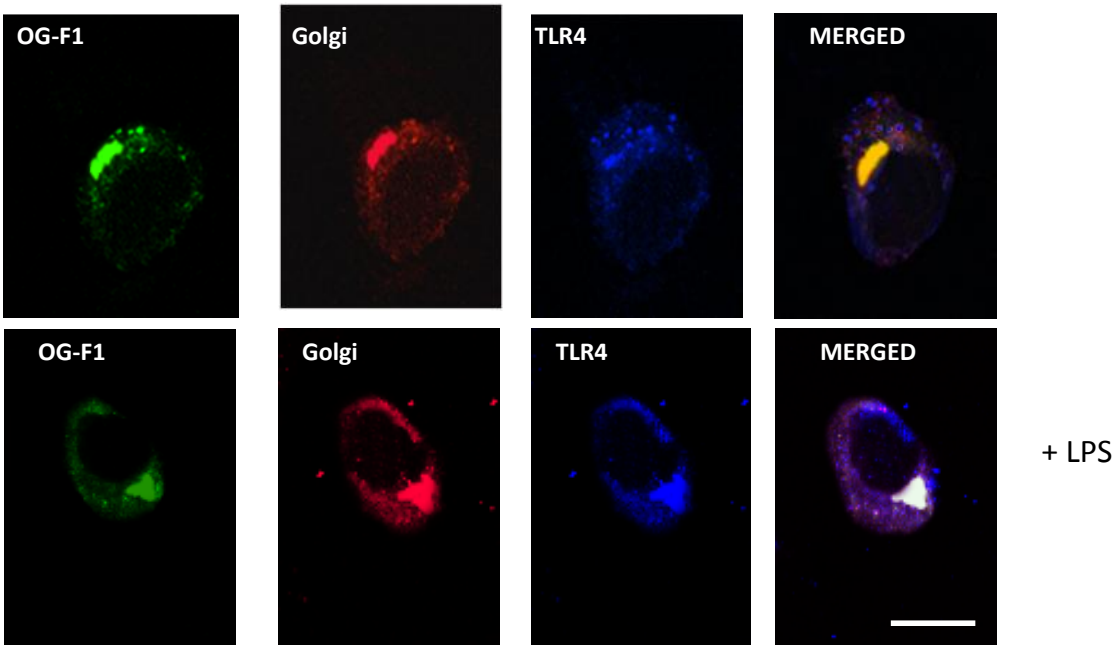


**Figure 8.7 – F1 antigen and TLR4 co-localise in the Golgi in response to LPS.** Intracellular distribution of F1 antigen and TLR4. F1 antigen labelled with Oregon-Green (OG-F1) (green) and TLR4 (blue) co-localise within the Golgi apparatus (red) in response to LPS. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.7. Intracellular co-localisation of F1 antigen and TLR2 in the presence and absence of LTA

Since F1 antigen was shown to be targeted to the Golgi apparatus along with TLR4 in response to LPS, the question was explored whether this was also the case with TLR2 in response to LTA.

In the absence of LTA (Figure 8.8, top panels) it was shown that F1 antigen co-localised with TLR2 within the Golgi apparatus. Upon LTA stimulation, TLR2 became more prominent along with F1 antigen in the Golgi apparatus (Figure 8.8, lower panels), suggesting that F1 antigen must interact with TLR2 on the cell surface in the presence of LTA and subsequently is targeted to the Golgi apparatus.

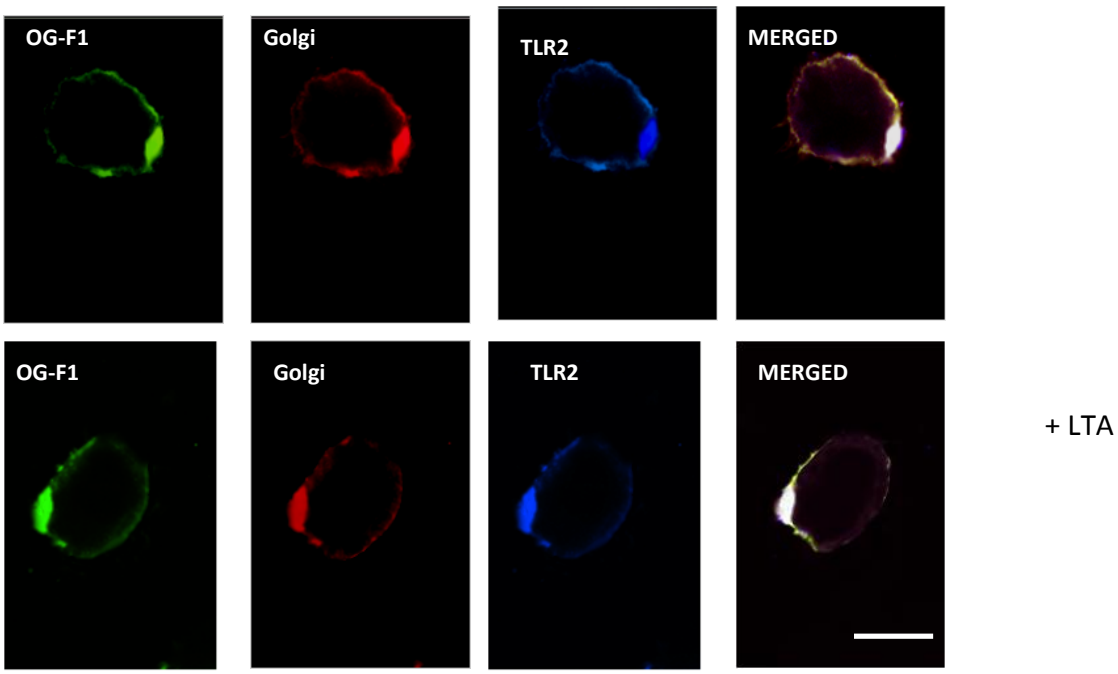


**Figure 8.8 – F1 antigen and TLR2 co-localise within the Golgi apparatus in response to LTA.** Intracellular distribution of F1 antigen and TLR2 in the presence (lower panels) and absence (top panels) of LTA. F1 antigen labelled with Oregon Green (F1-OG) (green) and TLR2 (blue) co-localise with the Golgi (red) in the presence and absence of LTA. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.8. MyD88 activation in the presence and absence of F1 antigen

Since F1 antigen had been shown to modulate TLR responses, the question that arose was whether it also interfered with TLR-induced signalling. Mono Mac 6 cells were incubated with either 100 ng/ml LPS (Figure 8.9) or 10 μg/ml LTA (Figure 8.10) for 30 min in the presence or absence of F1 antigen. Following stimulation the cells were fixed and the Golgi apparatus was labelled with an antibody against GM130, a protein within the Golgi apparatus, whereas TLR4 and TLR2 were labelled with a TLR-specific antibodies and the appropriate secondary antibody conjugated to Cy5. MyD88, the signalling adaptor molecule for both TLR2 and TLR4, was labelled with an antibody conjugated to FITC.

Confocal microscopy revealed that in the presence of either LPS or LTA, MyD88 co-localised with TLR4 or TLR2 respectively, suggesting that activation had occurred (Figure 8.9 & 8.10, top panels). In contrast, when the cells were stimulated in the presence of F1 antigen, TLR2 and TLR4 were still targeted to the Golgi apparatus but did not co-localise with the signalling molecule, MyD88 (Figure 8.9 & 8.10, lower panels), therefore suggesting that similarly to V antigen, the presence of F1 antigen inhibits MyD88 recruitment to TLRs, and thus abrogates signalling.

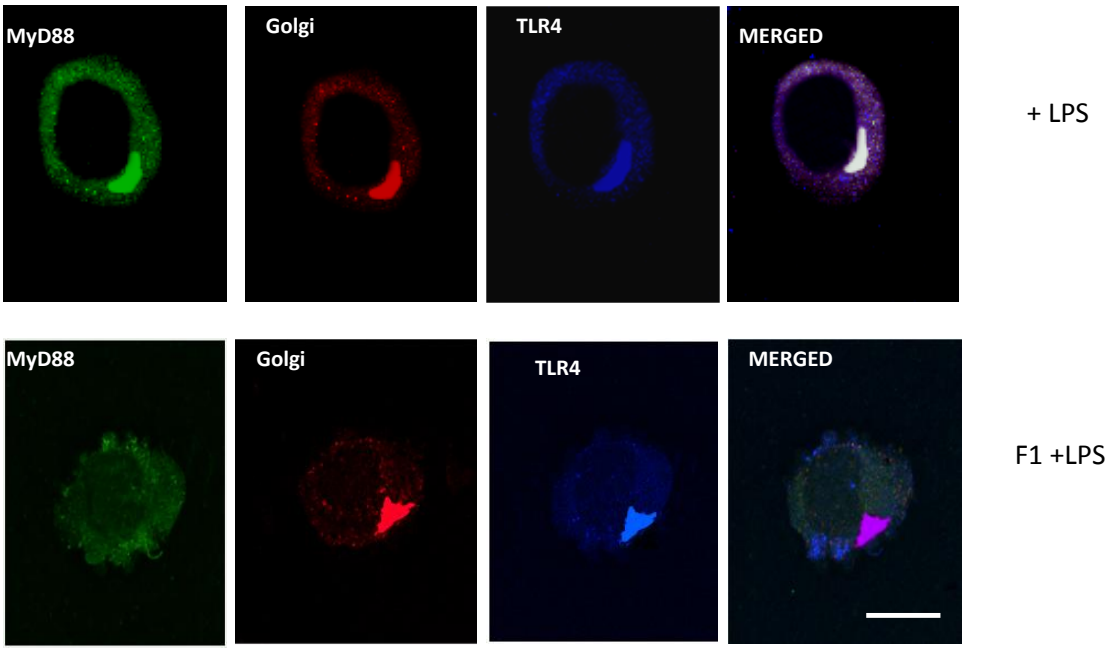


**Figure 8.9 – LPS-induced MyD88 recruitment in the presence and absence of F1 antigen.** Intracellular distribution of TLR4 and MyD88 in response to LPS (top panels) or LPS in the presence of F1 antigen (lower panels). TLR4 (blue) co-localised with MyD88 (green) in the Golgi apparatus (red) in response to LPS. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

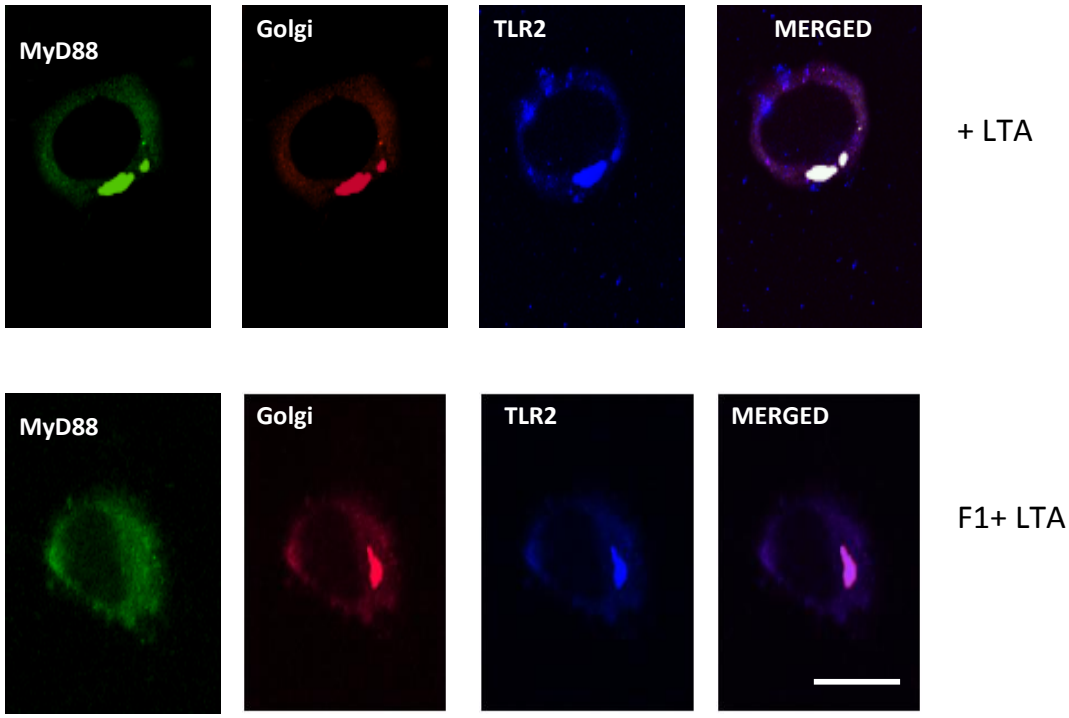


**Figure 8.10 – LTA-induced MyD88 recruitment in the presence and absence of F1 antigen.** Intracellular distribution of TLR2 and MyD88 in response to LTA (top panels) or LTA in the presence of F1 antigen (lower panels). TLR2 (blue) co-localised with MyD88 (green) in the Golgi apparatus (red) in response to LTA. Images were collected using a Zeiss 510 META confocal microscope. Scale Bar, 10 μm.

## 8.9. Conclusions

Most TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) activate cells by engaging their ligands on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 trigger signalling intracellularly. These TLRs have been shown to reside in the ER and to recognise their ligands once they have been endocytosed (Heil, et al., 2003; Nishiya & DeFranco, 2004).

TLR4 is the best studied TLR, and has been found to be recruited to lipid rafts upon stimulation by LPS (Triantafilou, et al., 2002), and to be targeted to the Golgi apparatus (Latz, et al., 2002). TLR2 has also been found to reside in lipid rafts after stimulation by Gram-positive bacterial products and to be similarly targeted to the Golgi apparatus (Triantafilou, et al., 2004). Thus it seems that once TLRs are engaged on the cell surface, they internalize along with their ligand and they are targeted to intracellular compartments.

Since *Y. pestis* V and F1 antigens have been shown to associate with TLRs and to modulate their signalling cascade it is possible that these proteins also affect receptor internalization as well as their intracellular trafficking and targeting. Confocal microscopy was used in order to investigate the intracellular trafficking and targeting of V and F1 antigens as well as TLRs in the presence and absence of their ligands.

Mono Mac 6 cells, which are a monocytic cell line, were incubated with either fluorescent V or F1 antigen for 30 min in the presence and absence of different TLR ligands (mainly LPS or LTA). Subsequently the cells were fixed and labelled using antibodies against specific TLRs and intracellular compartments.

It was shown that in the absence of one of the TLR ligands, V and F1 antigen were concentrated in the Golgi apparatus and co-localised there with TLR2 but not TLR4. When the cells were stimulated with the corresponding ligand, the V and F1 antigens concentrated in the Golgi along with the respective TLR molecule. The co-localisation of either V or F1 antigen with TLR2 regardless of the presence of the TLR2 ligand LTA suggests that V and/or F1 antigen associates with TLR2, possibly on the cell surface and are then internalised along with TLR2 and targeted to the Golgi apparatus. Indeed, this interaction between V antigen and TLR2 concurs with studies

that show this interaction occurring, including one study by Reithmeier-Rost and colleagues that tested the interaction between *Y. pestis* LcrV and TLR2 (Reithmeier-Rost, et al., 2007).

Most importantly, when it was investigated whether there was any recruitment of the signalling adaptor molecule MyD88 in the presence and absence of V or F1 antigens, it was shown that in the presence of the antigens, MyD88 was not recruited to the site where the TLR molecule was, therefore suggesting that V and F1 antigens associate with TLRs but interfere with PAMP-induced MyD88 recruitment, thus abrogating TLR signalling. This interference is clearly not due to either V or F1 antigen binding to MyD88 as when this interference occurs MyD88 is found away from the compartments where these bacterial proteins end up locating. It might, perhaps, bind to the TLRs themselves when these are brought into the Golgi apparatus. One way this might occur is with a conformational change forced on these receptors that changes their TIR domains' capability to bind to MyD88 or even by binding directly to the TIR domain of the TLRs, therefore blocking MyD88 binding.

# *Chapter 9 - Discussion*

*Yersinia pestis*, the aetiological agent of the plague, is a bacterium responsible for hundreds of millions of deaths throughout history. It invades the mammalian body, often through a flea bite, and proceeds to create a fulminating disease that tends to kill the host within a matter of days (Perry & Fetherston, 1997). To do this, *Y. pestis* encodes several virulence factors that modulate the host immune system. Two of these, also known to interfere with the generation of effective antibodies against them by the host's immune system, are V antigen (LcrV) and F1 antigen (Caf1) (Perry & Fetherston, 1997). V antigen has many functions, from being critical for gene regulation within the bacterium and for translocating other virulence factors (Yops) into host cells (Price, et al., 1991; Fields, et al., 1999; Derewenda, et al., 2004), to having immunomodulatory features of its own, mostly immunosuppressive (Leary, et al., 1995), such as inducing production of the anti-inflammatory cytokine IL-10 (Sing, et al., 2002; Reithmeier-Rost, et al., 2004). F1 antigen, whose fibers form a capsule-like structure on the outside of the bacterium, is best known for its antiphagocytic abilities, but it has also been shown to have immunomodulatory features of its own, mostly immunostimulatory.

In this study, it was decided to view the effects of these two antigens on the innate immune system specifically on human monocytic cells. Mono Mac 6 cells were used as a model monocytic cell line. Mono Mac 6 is the only myeloid cell line to constitutively express phenotypic and functional features of mature monocytes (Ziegler-Heitbrock, et al., 1988). Mono Mac 6 cells have several features of mature blood monocytes such as CD14 antigen expression, phagocytotic ability, and the functional ability to produce cytokines. This line is often used as an *in vitro* model to demonstrate the actions of monocytes (Neustock, et al., 1993), which is ideal for the present study.

The innate immune system is the host's quick response mechanisms to invasion by pathogens. Among the many mechanisms available to this division of the immune system is the pattern recognition receptors (PRRs), which are able to recognize certain motifs, particularly available in pathogens but not in the host. Among these PRRs, the most studied, and considered the most important, are the Toll-like receptors (TLRs). TLRs are expressed on immune cells and are able to distinguish a great variety of microbial ligands, such as cell wall components like lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria,

bacterial flagellin, CpG DNA, as well as viral DNA or single stranded RNA. All identified TLRs are type I transmembrane proteins, whose intracellular domains contain regions homologous to the intracellular domains of IL-1R and are referred to as TIR domains (Takeda, et al., 2003). These intracellular domains are able to trigger signalling pathways known to activate the nuclear factor kappa B (NF-κB) (Medzhitov, et al., 1998; O'Neill, 2000), which in turn leads to the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8.

TLR activation typically triggers a signalling cascade that results in alterations in gene expression and secretion of cytokines, amongst other things, to promote the appropriate response against the detected pathogens. Some pathogens, however, such as *Y. pestis*, are capable of subverting these mechanisms for their own survival, and it was their ability to modulate the innate immune system that was the focus of this study

In order to determine the effects of V and F1 antigens on the innate immune response, initially preparations of both antigens were generated. The *E. coli* strain containing the plasmid pVG110, encoding recombinant LcrV with a glutathione S-transferase (GST) tag, as well as the strain containing the plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain MP6, were kindly provided by Prof. Richard Titball (Chemical and Biological Defense Establishment, Porton Down, Salisbury, UK). The plasmid pVG110 was transformed into *E. coli* BL21 cells, as previously described (Carr, et al., 2000) while the plasmid pAH34L was transformed into *E. coli* JM101 cells, as previously described (Miller, et al., 1998).

Following purification, both recombinant proteins were passed through a Profos Endotrap® blue 10 (Hycult Biotechnology) to ensure there was no LPS in the final solution and this was verified using the *Limulus* amoebocyte lysate (LAL) assay, which did not detect any traces of LPS in the purified preparations. In addition, the molecular weights of the proteins were determined by SDS-PAGE and western blotting. V antigen was found to be 38 kDa, which is the predicted molecular weight of native V antigen, whereas for F1 there was one strong staining band around 21 kDa. Some lower molecular weight proteins were observed after Coomassie staining in the case of V antigen, which could be V antigen fragments. In the case of F1, there were higher

molecular weight proteins observed, which are probably F1 aggregates, since F1 is known to form multimers (Miller, et al., 1998; Tito, et al., 2001; Zavialov, et al., 2003).

Having shown the purification of the recombinant proteins, the effects of recombinant V and F1 antigens on Mono Mac 6 cells was then investigated. Initially the effect that V and F1 antigens had on TLR expression was studied. Mono Mac 6 cells were stimulated with different concentrations of V or F1 antigens over a 24 hour period. The cells were fixed and the TLR expression levels were determined by indirect immunofluorescence and flow cytometry. It was shown that both antigens were able to modulate the expression of PRRs, in particular TLRs 1 through 9 and also CD36, a scavenger receptor and known co-receptor of TLR2.

V and F1 antigens induced downregulation of all TLR expression, except TLR3, 1 hour after stimulation. This downregulation seemed to last for up to 4 hours in the case of V antigen, at which point TLR expression levels appeared to attempt to return to normal (‘basal’) levels. In order to verify whether the downregulation observed was dose-dependent, three different concentrations of V or F1 antigen (5 µg, 50 µg, 100 µg) were used. It was shown that throughout the different concentrations used, V and F1 antigen were still able to downregulate TLR expression. This could be an evasion strategy that *Y. pestis* employs in order to dampen the innate immune response with the secretion of V and/or F1 antigen and then infect the host cells without any resistance.

In cells stimulated with 5 µg of V antigen, the results show an “up-and-down” pattern of expression levels in which downregulation and upregulation of expression appears to alternate at each time point collected. Given that expression levels decrease below the basal levels when downregulation occurs and increase above basal levels when upregulation occurs, it is possible that these results indicate that, after the initial downregulation observed, the cells attempt to return TLR expression levels to normal but keep coming into contact with V antigen that dampens the response and thus forces the receptor downregulation observed..

This pattern was not observed with the slightly higher concentrations of V antigen used with other cells (50 µg and 100 µg). It is possible that the higher concentrations of V antigen might induce a different pattern of TLR expression, such as,

for example, repressing the upregulation of TLR expression, resulting in a slower and more prolonged increase in expression in an attempt by the cells to return to a basal state.

In contrast to what is observed with V antigen, the interaction with F1 seemed to induce a more sustained downregulation lasting throughout the 24 hour period of the experiment.

In contrast to the other TLRs, TLR3 showed a tendency for upregulation following V or F1 stimulation, possibly because it signals through a MyD88-independent pathway as opposed to the MyD88-dependent pathway that all the other TLRs use, suggesting an influence of V and F1 antigen on the MyD88-dependent pathway, and thus the TRIF pathway remains unaffected and tries to compensate by upregulating TLR3 and thus MyD88-independent signalling.

Similarly to TLR3, CD36, which is a scavenger receptor that helps bind and internalise pathogens, was also upregulated after stimulation with V or F1 antigen. This upregulation might possibly be part of a mechanism of trying to clear the bacteria found outside the cells. It might be an attempt to internalise *Y. pestis* within the cells and in order to degrade them. It is also interesting to note that the pattern of CD36 expression levels observed tends to be very similar to that of TLR3, regardless of which bacterial protein was used, suggesting, perhaps, a link between the two receptors or, at least, in the way they are affected by V or F1 antigen stimulation.

These findings appear to be in contrast to the studies of Sharma et al. (Sharma, et al., 2004; Sharma, et al., 2005) and Reithmeier-Rost et al. (Reithmeier-Rost, et al., 2004) where they observed upregulation of TLR2 and TLR5 in the former and no difference in TLR2 or TLR4 expression following V antigen stimulation in the latter study. Differences observed between the different studies could be due to the fact that the Sharma et al study was performed in Balb/c mice peritoneal macrophages whereas this study was performed in a human monocytic cell line and they were observing effects after 12 hours after stimulation. In the case of Reithmeier-Rost study, the strain of Yersinia used is completely different, as they used *Y. enterocolitica* O8 rLcrV, whereas in this study *Y. pestis* LcrV was used. It has been previously shown that LcrV

from *Y. enterocolitica* and LcrV from *Y. pestis* can have different effects from each other (Sing, et al., 2002; Reithmeier-Rost, et al., 2004; Abramov, et al., 2007).

Thus, it seems that V and F1 antigens might induce downregulation of TLR expression as an innate immune evasion mechanism causing immunosuppression during *Y. pestis* infection. The upregulation of CD36 might be a futile attempt by the host cell to promote internalisation of *Y. pestis*, given that F1 antigen is well-known to have an antiphagocytic property (Miller, et al., 1998; Salyers & Whitt, 2002; Liu, et al., 2006; Du, et al., 2002).

Having shown the effect of V and F1 antigen on TLR expression the next step was to determine how these proteins influenced NF-κB activation. This was assayed by measuring the intracellular concentration of phosphorylated IκB. It was shown that, during a 24 hour period, V and F1 antigens induced some phosphorylation of IκB, indicative of NF-κB activation, with the highest activation observed 8 hours after initial stimulation. This activation, however, occurred at very low levels in the first hours after initial stimulation and steadily increased from there.

Since the hypothesis was put forward that these antigens might interfere with the innate immune response, and thus cause immunosuppresion during *Y. pestis*, infection, the next step was to determine whether pre- or post-treatment with these antigens could alter the innate immune response to known PAMPs. The results suggested that either pre- or post-treament with V or F1 antigens inhibited phosphorylation of IκB and therefore, NF-κB activation. This inhibition was more prominent for the samples stimulated with either ssRNA, CpG DNA or Poly:I-C. These findings are in agreement with the study of Sodhi et al. (Sodhi, et al., 2004), in which they have also observed suppression of NF-κB activation in response to V antigen.

The low activation witnessed in the first hours after stimulation with only V or F1 antigen combined with these latter results suggests that perhaps these bacterial proteins trigger suppression of NF-κB in the first hours of contact with the monocytes. This suppression slowly tapers off and activation of NF-κB increases as the hours go by, with the highest amount of activation observed in this study occurring 8 hours after stimulation.

Since NF-κB activation was shown to be inhibited in the presence of V or F1 antigens, the next step was to investigate the effect of these proteins on cytokine secretion. Using the Th1/Th2 cytokine bead array from Becton Dickinson the secretion of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α was assayed over a 24h period. When it was investigated whether V or F1 antigens were able to trigger any cytokine response on their own, it was shown that the only cytokine that they could trigger was the anti-inflammatory IL-10. The lack of TNF-α secretion contradicts the study of Reithmeier-Rost and colleagues but the secretion of IL-10 is in agreement with their study (Reithmeier-Rost, et al., 2004), suggesting that these proteins are able to trigger anti-inflammatory cytokine production and thus suppress the innate immune response. The secretion of IL-10, however, was first observed only 8 hours after stimulation with either V or F1, coinciding with the observed maximum activation of NF-κB. It is possible, perhaps, that this large presence of activated NF-κB triggers production of IL-10. As monocytes do not secrete IL-2, IL-4 or IFN-γ, the lack of secretion shown by the assay merely served to confirm this. The cytokines TNF-α and IL-6, however, are secreted by monocytes, as shown in samples of cells stimulated with known PAMPs. That stimulation with only V or F1 does not cause any secretion of either of these two inflammatory cytokines shows the immunosuppressive effect of these two proteins. It also shows that IL-10 secretion is not occurring due to TNF-α secretion as observed by Reithmeier-Rost and colleagues (Reithmeier-Rost, et al., 2004).

Pre- or post-treatment with V or F1 antigens in response to known PAMPs was also tested. It was shown that V and F1 antigen downregulated secretion of TNF-α, IL-6 and IL-10, thus suggesting that these proteins induce a tolerance effect and inhibit the secretion of cytokines triggered by known PAMPs, including the anti-inflammatory IL-10. Together with the previous observations seen regarding NF-κB activation being suppressed by V and F1 antigen, this once again suggests the possibility that this decrease of cytokine secretion is linked to suppression of NF-κB activation.

Having determined the immunomodulating effects of both V and F1 antigens, the next step was to investigate whether these antigens internalize and what is their intracellular trafficking and targeting within the cells. In order to track the antigens within the cells, V and F1 recombinant proteins were conjugated with a fluorescent tag,

Oregon Green (OG). Mono Mac 6 cells were incubated with V- or F1-OG and subsequently fixed and labelled for particular intracellular organelles. Both seemed to be targeted to an intracellular location, which was thought to be the Golgi apparatus. In order to confirm that indeed it was the Golgi apparatus, cells were labelled with an antibody against GM130, which is a cis-Golgi matrix protein (Nakamura, et al., 1995).

It was shown that upon V- or F1-OG stimulation with Mono Mac 6 cells, the proteins were internalised and within 30 min were localised in the Golgi apparatus. When it was investigated whether they co-localised there with TLR receptors, in particular TLR2 and TLR4, it was shown that, if cells were not treated with a respective PAMP known to stimulate these TLRs (LTA for TLR2 and LPS for TLR4), there was co-localisation of the *Yersinia* proteins with TLR2 but no co-localisation of these proteins with TLR4. Both *Yersinia* proteins as well as TLR2 were found to co-localise within the cis-Golgi while TLR4 was located in the endosomes. Upon stimulation with their respective PAMPs, TLR4 and TLR2 co-localised with V or F1 antigens within the Golgi apparatus. Therefore, suggesting that upon stimulation with bacterial ligands, *Yersinia pestis* antigens are targeted to the Golgi along with the TLRs, as a possible clearance mechanism. These data are in agreement with previous studies that have shown that TLR4 (Latz, et al., 2002) and TLR2 (Triantafilou, et al., 2004) are targeted to the Golgi in response to their respective ligands. As V and F1 antigen co-localised in the Golgi along with TLR2 even without the presence of LTA, this suggests the possibility that the *Yersinia* proteins bind to TLR2 and are therefore internalised along with it. In the case of V antigen, this is supported by previous studies showing a link to TLR2, such as that performed by Reithmeier-Rost and colleagues (Reithmeier-Rost, et al., 2007).

The question that remained was whether this internalization event was linked with signalling, especially given the experiments studying TLR expression and the results showing that the expression of TLRs utilising the MyD88-dependent pathway was downregulated but not the expression of the only TLR that does not utilise this pathway (TLR3). In order to answer this question, the cells were stimulated with LPS or LTA in the presence and absence of V or F1 antigens and the recruitment of MyD88 was investigated. It was shown that, in the absence of V or F1 antigens, TLR4 or TLR2

co-localised with MyD88, thus showing that there was recruitment and activation of signalling. In the presence of V or F1 antigens, TLR4 or TLR2, in response to their ligands, no longer co-localised with MyD88, thus signalling was inhibited. Therefore supporting the hypothesis that indeed V and F1 antigens interfere with the MyD88-dependent signalling cascade leading to NF-κB activation. This interference appears to be done by preventing binding of MyD88 to the TLRs. One hypothesis on the mechanism causing this effect could be that these bacterial proteins are binding to the TLRs when they happen to co-localise in the same compartment, perhaps by binding to the part of the TIR domain recognized by MyD88.

This project has yielded results that show that both V and F1 antigen possess immunosuppressive effects. Both *Yersinia pestis* proteins induce a downregulation of TLR expression (with the exception of TLR3) as well as suppressing NF-κB activation in the first hours after stimulation and, therefore, suppressing cytokine secretion during these early hours, possibly by a mechanism in which these bacterial proteins prevent MyD88 from binding to the TLRs that utilise the signalling pathway dependent on this adaptor molecule. After this first period, the suppressive effect on NF-κB activation has subsided and secretion of the anti-inflammatory IL-10 occurs, which likely suppresses secretion of the other inflammatory cytokines.

This is the first study to have investigated in-depth the effects of V and F1 antigens on TLR expression and activation. In addition, it is the first study, to demonstrate that these antigens interfere with the MyD88-signalling cascade, thus inhibiting the innate immune response. It would be of interest to investigate these findings in the context of a true *Y. pestis* infection.

## 9.1. Possible future work

Since both V and F1 antigens have been shown to have immunomodulatory activity and are able to inhibit inflammatory cytokine secretion, it would be of great interest in the future to use them as therapeutic interventions for inflammatory conditions. In particular since post-treatment with both antigens has proven to inhibit inflammatory secretion one would expect that they would be suitable as an anti-inflammatory treatment in diseases such as sepsis, rheumatoid arthritis, atherosclerosis, etc. Therefore it would be of interest to be tested in animal models of inflammatory diseases in the future.

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# *APPENDIX*

## RECIPES

### Luria-Broth

| | 1 L | 5 L |
|---|---|---|
| Bacto peptone | 10 g | 50 g |
| Yeast extract | 5 g | 25 g |
| NaCl | 5 g | 25 g |

### 10x Phosphate-Buffered Saline (PBS) (1 L)

| | |
|---|---|
| NaCl | 100 g |
| KCl | 2.5 g |
| $Na_2HPO_4$ ($Na_2HPO_4$ $2H_2O$) | 14.4 g (18.0 g) |
| $KH_2PO_4$ | 2.5 g |
| Distilled water | Up to 1000 ml |
| pH to 7.2 with HCl if needed | |

### 1x Phosphate-Buffered Saline (PBS) (500 ml)

Dilute 50 ml of 10x PBS to 500 ml of 1x PBS with distilled water

### 0.01% (w/v) Triton X-100

Add 0.01 g per 100 ml of distilled water

**Cleavage Buffer, pH 7 (250 ml)**

| | |
|---|---|
| 50 mM Tris | 1.514 g Tris |
| 150 mM NaCl | 2.19 g NaCl |
| 1 mM EDTA | 0.073 g EDTA |
| 1 mM DTT | 0.0385 g DTT |
| 0.01% (w/v) Triton X-100 | 25 µl (or 0.025 g of Triton X-100) |
| Distilled water | Up to 250 ml |
| pH to 7 with HCl | |

**1x PBS/1% Triton X-100 (w/v)**

Add 1 g of Triton X-100 per 100 ml of 1x PBS

**1x PBS/3 M NaCl (500 ml)**

| | |
|---|---|
| NaCl | 87.66 g |
| 1x PBS | Up to 500 ml |

**20% (v/v) Ethanol (100 ml)**

| | |
|---|---|
| Ethanol | 20 ml |
| Distilled water | 80 ml |

**6 M Guanidine HCl (500 ml)**

| | |
|---|---|
| Guanidine | 286.59 g |

**Standard coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl), pH 8.3 (500 ml)**

Mw($NaHCO_3$) = 84.01 g/mol

Mw(NaCl) = 58.44 g/mol

| | |
|---|---|
| $NaHCO_3$ | 8.401 g |
| NaCl | 14.61 g |
| Distilled water | Up to 500 ml |
| pH to 8.3 with NaOH | |

**Buffer A (0.5 M ethanolamine, 0.5 M NaCl), pH 8.3 (500 ml)**

Mw($C_2H_7NO$) = 61.08 g/mol

Mw(NaCl) = 58.44 g/mol

| | |
|---|---|
| Ethanolamine ($C_2H_7NO$) | 15.27 g |
| NaCl | 14.61 g |
| Distilled water | Up to 500 ml |
| pH to 8.3 with HCl | |

**Buffer B (0.1 M sodium acetate, 0.5 M NaCl), pH 4 (500 ml)**

Mw($NaC_2H_3O_2$) = 61.08 g/mol

Mw(NaCl) = 58.44 g/mol

| | |
|---|---|
| Sodium acetate ($NaC_2H_3O_2$) | 4.1015 g |
| NaCl | 14.61 g |
| Distilled water | Up to 500 ml |
| pH to 4 with HCl | |

**Trifluroacetic acid (TFA) elution buffer (0.1% TFA/1x PBS), pH 5**

Dissolve 0.1 g of TFA for every 100 ml of 1x PBS (0.5 g TFA for 500 ml PBS) and pH to 5

**2x PBS (250 ml)**

Dilute 50 ml of 10x PBS to 250 ml of 2x PBS with distilled water

**4% paraformaldehyde (PFA) (500 ml)**

In a fume hood:

- Add 20.0 g of paraformaldehyde to 250 ml of distilled water
- Heat to 60$^{o}$C
- Add a few drops of 1 M NaOH until the solid dissolves
- When the solid has completely dissolved, let solution cool and then add 250 ml of 2x PBS

**PBS/0.02% BSA/0.02% $NaN_3$/0.02% Saponin**

For every 100 ml of 1x PBS add 0.02g of BSA, 0.02g of sodium azide ($NaN_3$) and 0.02g of saponin

**SDS-PAGE Fixing solution (2 L)**

| | |
|---|---|
| Acetic acid | 200 ml |
| Absolute ethanol | 800 ml |
| Distilled water | 1000 ml |

**Commassie Blue stain (200 ml)**

| | |
|---|---|
| Methanol | 100 ml |
| Acetic acid | 20.0 ml |
| Distilled water | 80.0 ml |
| Commassie Blue | 0.6 g |

**Destain (1 L)**

| | |
|---|---|
| Ethanol | 100 ml |
| Acetic acid | 100 ml |
| Distilled water | 800 ml |

**0.5 M Tris-HCl pH 6.8 (100 ml)**

| | |
|---|---|
| Tris | 6.0 g |
| Distilled water | Up to 100 ml |
| pH to 6.8 with HCl | |

**2x SDS-PAGE Reducing Buffer [125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 1.4 M β-mercaptoethanol] (20 ml)**

| | |
|---|---|
| 0.5 M Tris, pH 6.8 | 10 ml |
| 10% SDS stock | 8.0 ml |
| Glycerol | 5.0 g |
| 14.3 M β-mercaptoethanol | 2.0 ml |
| Bromophenol Blue | 0.5 mg |

**1.5 M Tris-HCl pH 8.8 (100 ml)**

| | |
|---|---|
| Tris | 18.17 g |
| Distilled water | Up to 100 ml |
| pH to 8.8 with HCl | |

**Resolving gel**

| **Acrylamide %** | **10%** | **12%** |
|---|---|---|
| Distilled Water | 4.02 ml | 3.5 ml |
| 1.5 M Tris-HCl pH 8.8 | 2.5 ml | 2.5 ml |
| 10% (w/v) SDS | 100 µl | 100 µl |
| Acrylamide/Bis | 3.33 ml | 4.0 ml |
| 10% APS | 50.0 µl | 50.0 µl |
| TEMED | 5.0 µl | 5.0 µl |

**4% Stacking gel**

| | **One Gel** |
|---|---|
| Distilled Water | 3.05 ml |
| 0.5 M Tris-HCl pH 6.8 | 1.25 ml |
| 10% (w/v) SDS | 50.0 µl |
| Acrylamide/Bis | 0.65 ml |
| 10% APS | 50.0 µl |
| TEMED | 10.0 µl |

**SDS-PAGE Running Buffer (1 L)**

| | **x1** | **x10** |
|---|---|---|
| Tris | 3.0 g | 30.0 g |
| Glycine | 14.4 g | 144 g |
| SDS | 1.0 g | 10.0 g |

**Transfer Buffer (1 L)**

| **Reagents** | **x1** | **x2** |
|---|---|---|
| 20 mM Tris-acetate | 2.44 g Tris | 4.88 g Tris |
| 0.1% SDS | 10 ml of the 10% stock | 20 ml of the 10% stock |
| 20% isopropanol | 200 ml isopropanol | 400 ml isopropanol |
| pH to 8.3 with acetic acid | | |
| | | |
| **Final Volume** (add distilled water) | 1000 ml | 1000 ml |

**PBS-Tween (1 L)**

For every 1000 ml of 1x PBS, add 1 ml of Tween-20

**Blocking solution**

Add 2 g of milk powder per 10 ml of PBS-Tween

**Stripping buffer**

| | |
|---|---|
| 100 mM mercaptoethanol | 1.4 ml |
| 2% SDS | 40 ml from the 10% stock |
| 62.5 mM Tris-HCl | 1.52 g Tris |
| pH to 6.7 with HCl | |
| | |
| **Total Volume** (add distilled water) | 200 ml |

**1 M $NaHCO_3$, pH 8 (100 ml)**

| | |
|---|---|
| $NaHCO_3$ | 8.4 g |
| Distilled water | Up to 100 ml |
| pH to 8 | |

**Alcoholic HCl (100 ml)**

| | |
|---|---|
| Ethanol | 70 ml |
| 18 M concentrated HCl | 6.0 ml |
| Distilled water | 24 ml |

**0.01% Poly-l-lysine (100 ml)**

| | |
|---|---|
| 0.1% (w/v) Poly-l-lysine stock | 1 ml |
| Distilled water | Up to 100 ml |

# ***LIST OF FIGURES***

# ***LIST OF TABLES***